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**Determination of Anticoagulant Rodenticides and  $\alpha$ -Chloralose in Human Hair. Application to a Real Case**

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**DETERMINATION OF ANTICOAGULANT RODENTICIDES AND  
 $\alpha$ -CHLORALOSE IN HUMAN HAIR AND APPLICATION TO A  
REAL CASE**

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1 DETERMINATION OF ANTICOAGULANT RODENTICIDES AND  $\alpha$ -CHLORALOSE IN HUMAN HAIR  
2 APPLICATION TO A REAL CASE

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**ABSTRACT**

Anticoagulant rodenticides are the largest group of poisons used to kill harmful rodents. Their fundamental mode of action consists in the inhibition of the vitamin K epoxide reductase, which causes blood-clotting alteration, ultimately leading to hemorrhagic events as the cause of death. In this study, we developed an UHPLC-MS/MS for the simultaneous determination of 10 anticoagulant hydroxycoumarine rodenticides, plus  $\alpha$ -chloralose in human hair, with the scope of detecting potential trace of chronological poison exposure in clinical and forensic cases. The method was fully validated and applied to a case of intentional poisoning perpetrated by administration of difenacoum and  $\alpha$ -chloralose to a 97-years old woman, who was hospitalized because of severe symptoms, including drowsiness, convulsions, pallor and hematoma. Hair sample from the victim was segmentally analyzed. and proved sensitive enough to detect occasional exposure of the victim to the two analytes. Difenacoum was detected in the proximal 3-cm hair segment at the concentration of 2.9 pg/mg. To our knowledge, this is the first study to report that exposure to difenacoum is detectable in real hair samples. The other target analyte found in the hair sample was  $\alpha$ -chloralose, which was detected in the 0-3 cm segment at the concentration of 85 pg/mg. The two subsequent and consecutive segments (3-6 cm and 6-9 cm) showed only traces of difenacoum (below LOQ) and low but quantifiable levels of  $\alpha$ -chloralose (29 pg/mg and 6 pg/mg, respectively). Therefore, hair segmental analysis allowed us to conclude that the victim was repeatedly exposed to two poisons in the period corresponding to the first segment of hair.

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**KEYWORDS:** UHPLC-MS/MS; hair; anticoagulant rodenticides;  $\alpha$ -chloralose; poisoning

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## INTRODUCTION

Anticoagulant rodenticides are the largest group of poisons used to kill harmful rodents. They are classified depending on their chemical structure into two main groups: hydroxycoumarine and indandione rodenticides. The chemical structure of anticoagulant compounds is related to that of natural compounds such as ferulenol or dicoumarol, the latter being found in moldy sweet clover. Their action mechanism is almost identical, even when they are used within therapeutic protocols, producing similar clinical effects, hematological changes and treatment schedule, regardless of their specific structural identity (1). Their fundamental mode of action consists in the inhibition of the vitamin K epoxide reductase, which causes blood-clotting alteration, ultimately leading to hemorrhagic events as the cause of death (2). The first marketed product (warfarin) has been continuously used as either a rodenticide or a therapeutic drug to prevent thromboembolic disorders in human beings. The first generation of anticoagulants (warfarin, coumatetralyl, coumachlor, diphacinone, and chlorophacinone) produce their effect in rodents only after several days of feeding (3). The massive use of warfarin resulted in the development of resistance in several strains of rodents (4). As a consequence, newer and more active products were developed to overcome these problems, such as brodifacoum and flocoumafen. These second generation anticoagulants are active after a single intake. The widespread use of anticoagulant rodenticides led to the need of analytical methods to monitor accidental or intentional intoxication of either domestic animals or humans (5-7). In the past, a number of techniques have been used to detect anticoagulant rodenticides, including gas chromatography-electron capture detection (58), gas chromatography-mass spectrometry (69), immunoassay (710), thin-layer chromatography-UV/fluorescence (811) and high-performance liquid chromatography (HPLC) with fluorescence and UV/photodiode array detectors (9-1012-13). In recent years, liquid chromatography coupled to mass spectrometry (LC-MS) has become the most frequently used technique for these analyses (11-1614-19). Besides anticoagulants,  $\alpha$ -chloralose is also commonly used as a rodenticide. It shows both a depressive effect on the central nervous system, producing sedation and anesthesia, and a stimulant effect on spinal reflexes, producing spontaneous myoclonic movements or generalized convulsions (17-1920-22).  $\alpha$ -chloralose is often found in specimens from animal autopsies, in particular in baits and bird organs. Furthermore, cases of accidental ingestion involving humans or intentional suicides have been reported (20-2223-25). Many studies about rodenticides detection in biological samples were published in the past (2326), but only one on human hair, limited to brodifacoum and bromadiolone (2427).

In clinical and forensic toxicology, blood, plasma, or serum concentrations of anticoagulant rodenticides are utilized to diagnose and predict the duration of vitamin K1 therapy, or to estimate a state of intoxication at the moment of sampling. On the other hand, the keratin matrix, namely hair, is commonly used to establish a potential chronological drug/poison exposure, with further periods corresponding to the hair segments

79 | more distant from the hair root (~~25-28~~28-31). Furthermore, hair analysis may provide a non-invasive  
80 | approach for monitoring previous exposure also for wild animals, with easier storage and longer detection  
81 | time compared to other non-invasive methods such as detection in pellets of bird of prey, or faeces of  
82 | mammals (2427).

83 | In the present study, we developed an UHPLC-MS/MS for the simultaneous determination of 10  
84 | anticoagulant rodenticides plus  $\alpha$ -chloralose in human hair. The method was fully validated and applied to  
85 | a case of intentional poisoning perpetrated by administration of difenacoum and  $\alpha$ -chloralose.

## 87 | **EXPERIMENTAL**

### 89 | **Chemicals, reagents, and standard solutions**Materials

90 | Coumatetralyl, brodifacoum, bromadiolone, difenacoum, flocoumafen, coumachlor, acenocoumarol,  
91 | coumafuryl, dicoumarol,  $\alpha$ -chloralose, methanol (MeOH), dichloromethane and ammonium formiate were  
92 | provided by Sigma-Aldrich (Milan, Italy). Warfarin was purchased by LGC (Sesto San Giovanni (MI), Italy)  
93 | and warfarin-d5 by C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Ultrapure water was obtained by a  
94 | Milli-Q Millipore system (Bedford, MA, U.S.A.). Stock standard solutions of analytes and warfarin-d5 as  
95 | internal standard (IS), were prepared in MeOH at a concentration of 200 mg L<sup>-1</sup> and stored at -20°C in the  
96 | dark. Working MeOH solutions containing all the analytes at different concentrations were prepared by  
97 | mixing the stock solutions at the proper dilution. The working solutions were used to spike negative hair  
98 | samples at various levels.

### 100 | **Analytical method** Instrumental conditions

101 | All analyses were performed on a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany)  
102 | interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany)  
103 | with an electrospray Turbo Ion source operating in the negative (ESI-) ion mode. A Kinetex C18 column  
104 | 50x2.1 mm i.d. x 1.7  $\mu$ m (Phenomenex, Italy), protected by a C18 guard column, was used for the  
105 | separation of analytes. The column oven was maintained at +40 °C, and the elution solvents used were  
106 | water/ammonium formiate 2 mM (solvent A) and MeOH (solvent B). The mobile phase eluted under the  
107 | following conditions (a/b; v/v): initial 80:20 ratio for 1 min, then linear gradient to 0:100 in 4 min; final  
108 | isocratic condition at 100% B for 0.5 min. The flow rate was 0.5 mL/min and total run time was 8.5 min,  
109 | including the re-equilibration time at the initial conditions between two consecutive injections. The MS  
110 | system was operated in the selected reaction monitoring mode (SRM). In order to establish appropriate  
111 | SRM conditions, each analyte was individually infused into the ESI capillary, while the declustering  
112 | potential (DP) and the entrance potential (EP) were adjusted to maximize the intensity of the [M-H]<sup>-</sup>  
113 | species. The collision offset voltage (CE) was adjusted to preserve approximately 10 % of the precursor ion,

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3 114 and the cell exit potentials (CXP) were also optimized. Each SRM transition was maintained during a time  
4 115 window of  $\pm 10.0$  s around the expected retention time of the corresponding analyte, and the SRM target  
5 116 scan time (i.e., sum of dwell times for each SRM cycle) was 0.30 s, including pause times of 5 ms between  
6 117 consecutive SRM transitions. The best results were obtained using a source block temperature of +500 °C  
7 118 and an ion-spray voltage of -3.000 V. Both Q1 and Q3 were operated at unit mass resolution. Nitrogen was  
8 119 employed as the collision gas at  $5 \times 10^{-3}$  Pa. The gas settings were as follows: curtain gas 30.0 psi, collision  
9 120 gas 8.0 psi, ion source gas GS1 40.0 psi, and ion source gas GS2 50.0 psi. The Analyst 1.5.2 (AB Sciex)  
10 121 software was used for data processing. The chemical structures of the anticoagulant rodenticides  
11 122 investigated in this study and  $\alpha$ -chloralose are reported in Figure 1. All analytes and IS, their corresponding  
12 123 retention time, SRM transitions, and potentials are presented in Table 1.  
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### 22 125 Case study

23 126 A 97-years old woman was hospitalized because of severe symptoms, including drowsiness, convulsions,  
24 127 pallor and hematoma. The laboratory tests showed abnormal values for coagulation parameters  
25 128 (prothrombin time-international normalized ratio PT-INR = 12.46; activated partial thromboplastin time  
26 129 aPTT = 60 s; aPTT ratio = 1.82). After intra-venous (IV) administration of 10 mg vitamin K and one day  
27 130 monitoring, the patient recovered from the hemorrhagic syndrome (PT-INR = 1.45; aPTT = 40.6 s; aPTT ratio  
28 131 = 1.23) and was dismissed. After one week, the woman was taken again to the Emergency Department (ED)  
29 132 because she accused the same symptoms. Her coagulations parameters were the following: PT-INR = 13.31;  
30 133 aPTT = 68 s; aPTT ratio = 2.06. After IV administration of vitamin K and three blood transfusions, the patient  
31 134 recovered once more.

32 135 During the treatment of the second episode, a blood sample was collected and screened for anticoagulants,  
33 136 in order to find possible explanations. Screening for further substances, which may account for convulsive  
34 137 symptoms, were not performed. Since the blood sample resulted positive to difenacoum, the case was  
35 138 reported to the Public Prosecutor's office, which took jurisdiction of the case. A fruit mousse allegedly used  
36 139 to poison the victim was seized by the Police. Our laboratory was asked by the Prosecutor to determine the  
37 140 content of the mousse and to estimate for how long the poisoning occurred. In order to respond to the  
38 141 latter query, the victim was asked to give a hair sample on which to perform the inherent toxicological  
39 142 analyses. The patient's hair sample was taken 2.5 months after her first hospitalization.  
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### 50 143 51 144 **Sample preparation**

52 145 About 200 mg of hair was twice-washed with dichloromethane (2 mL, vortex mixed for 3 min). After  
53 146 complete removal of solvent wash, the hair was dried at room temperature by a gentle nitrogen flow and  
54 147 subsequently cut with scissors into 1–2 mm segments. An aliquot of about 50 mg was weighted and then  
55 148 fortified with 62.5  $\mu$ L of warfarin-d5 an IS-working solution at 20 ng/mL, yielding a final concentration of 25  
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3 149 | pg/mg. [Similarly to the analysis of other xenobiotics in hair \(32\)](#), ~~sa~~sample extraction was carried out by  
4 150 | addition of 1 mL of methanol, vortex shaking for 5 min and centrifuging at 4000 rpm for 3 min, to ensure  
5 151 | the complete immersion of the matrix into the solvent, and final incubation at 55°C for 15 h. Lastly, the  
6 152 | organic phase was collected, and evaporated to dryness under a gentle stream of nitrogen and mild  
7 153 | heating (25°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was  
8 154 | dissolved in 100 µL of MeOH, transferred into a vial, centrifuged at 4000 rpm for 10 min. 2 µL of solution  
9 155 | was injected into the UHPLC–MS/MS system.  
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#### 16 157 **Validation**

17 158 | The analytical method was validated in accordance with the criteria and recommendations of international  
18 159 | standard and international guidelines ([2933](#)). The following parameters were investigated: specificity,  
19 160 | selectivity, linearity range, detection and quantification limits (LOD and LOQ), intra-assay and inter-assay  
20 161 | precision and accuracy. Carry-over and matrix effect were also investigated.  
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#### 24 162 25 163 *Specificity*

26 164 | A pool of five blank hair samples obtained from different healthy volunteers (two females, three males)  
27 165 | was prepared without spiking and analyzed as described above. The occurrence of possible interferences  
28 166 | from endogenous substances was tested by monitoring the SRM chromatograms characteristic for each  
29 167 | investigated compound at the expected retention time interval. The S/N was measured on the less intense  
30 168 | mass transition at the expected analyte retention time. The noise was measured from the end of the peak  
31 169 | till ±0.05 min after it for each analyte. A S/N<3 was considered satisfactory in order to verify the method's  
32 170 | specificity.  
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#### 40 171 41 172 *Selectivity*

42 173 | The repeatability of relative peak intensities for the transitions of each analyte was determined on five  
43 174 | spiked hair samples at two concentration levels (5 and 250 pg/mg). Retention time precision at each  
44 175 | concentration was also determined.  
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#### 48 176 49 177 *Linearity, LOD and LOQ*

50 178 | The linear calibration model was checked by analyzing (two replicates) blank hair samples spiked with the  
51 179 | working solution at five concentration levels (5, 25, 100, 250, 500 pg/mg). The calibration was completed  
52 180 | by internal standardization. The squared correlation coefficient, adjusted by taking into account the  
53 181 | number of observations and independent variables ( $\text{Adj}R^2$ ), was utilized to roughly estimate linearity.  
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56 182 | The limit of detection (LOD) was estimated with the Hubaux-Vos approach ([3034](#)). LOQs was then  
57 183 | calculated as 2 times the LOD values.  
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*Precision and accuracy*

For all analytes, intra- and inter-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias %) were evaluated at two concentration levels. Ten replicates of blank hair samples were spiked with the standard solutions at the lowest calibration point (close to LOQ values) and at intermediate calibration level, i.e. 5 and 250 pg/mg concentrations. Intra-assay precision was considered satisfactory when CV% values were below 15%. Satisfactory accuracy was achieved when the experimentally determined average concentration lied within  $\pm 15\%$  from the expected value.

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*Carry-over*

The background chromatographic profiles for each analyte were monitored during the analysis of blank hair sample injected for five times after the chromatographic run of a spiked blank hair sample containing all the analytes at 500 pg/mg concentration. To assure the absence of carry-over, the same criteria adopted to verify the specificity requirements had to be respected.

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*Matrix effect*

Matrix effect (expressed as percentage variation) was evaluated at 25 pg/mg analytes concentration by comparing the signals obtained when the analytes were added to the matrix extract with those acquired from a methanol solution containing the analytes at the same concentration. Since the quantification of real samples is performed by internal standardization, the 'relative' matrix effect, namely the effect calculated by comparing the peak areas of the analyte, divided by the peak area of the IS, both in matrix and methanol, was also evaluated. In the latter case, the matrix effect is expected to be partly compensated by the choice of a suitable internal standard, both analyte and IS undergoing similar interference from the matrix. The matrix effect was calculated as the mean of five replicates. The difference with respect to 100% highlighted matrix suppression (values below 100%) or enhancement (values above 100%) (~~3135~~).

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**RESULTS AND DISCUSSION**

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**Method development**

The optimized UHPLC-MS/MS method allowed the simultaneous determination of 10 anticoagulant rodenticides plus  $\alpha$ -chloralose. The whole chromatographic run, comprehensive of the time required for column re-equilibration, was completed in 8.5 min. Retention times ranged between 1.39 min (coumafuryl) and 4.33 min (brodifacoum). Figure 2 shows the SRM chromatograms recorded from a blank hair spiked at 5 pg/mg for all the analytes. These concentrations correspond to the lowest point of the calibration curves.

219 Two SRM transitions were selected for each analyte with the exception of dicoumarol for which only a  
220 transition is available.

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## 222 Validation

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### 224 *Specificity and Selectivity*

225 SRM chromatograms from negative hair samples showed no interfering signals at the retention time where  
226 the analytes were expected to elute. Thus, all specificity tests proved successful.

227 To assess selectivity, one qualifying transition was monitored, in addition to the primary fragmentation  
228 with the exception of dicoumarol (Table 1). Variations of relative qualifier ion intensities did not exceed  
229  $\pm 20\%$  with respect to the corresponding control and were considered acceptable.

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### 231 *Linearity, LOD and LOQ*

232 Table 2 reports the AdjR<sup>2</sup> values obtained from the calibration curves that range from 0.9926 up to 1.000  
233 and indicate good fit and linearity. LOD values ranged from 0.3 pg/mg for warfarin to 2.6 pg/mg for  
234 brodifacoum, while LOQ values lied between 0.6 pg/mg and 5.1 pg/mg. The calculated LODs were  
235 experimentally confirmed with five blank hair samples spiked at concentrations below these. As a matter of  
236 fact, LOD values calculated with the Hubaux-Vos method are quite conservative, since both CC $\alpha$  and CC $\beta$   
237 risks are minimized at 5%, making the LOD values significantly higher than the limits of decision.  
238 Consequently, the LOQ values were realistically calculated as 2  $\times$  LOD, instead of 3  $\times$  LOD. This choice is  
239 supported by the satisfactory precision and accuracy data obtained at 5.0 pg/mg, i.e. close or below the  
240 LOQ values calculated for  $\alpha$ -chloralose, acenocoumarol, and brodifacoum (see below).

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### 242 *Precision and accuracy*

243 Intra- and inter-day data on precision and accuracy are reported in Table 3. The results show satisfactory  
244 intra-day repeatability, as the percent variation coefficient (CV%) is lower than 10% for all the spiked  
245 analytes at low and high concentration, with only one exception (11% for dicoumarol at 250 pg/mg). The  
246 intra-day results also demonstrated optimal accuracy, as the percent bias are within few percent in almost  
247 all cases, with maximum experimental errors of -12% and +9.6%.

248 Also inter-day repeatability and accuracy results proved fully satisfactory, as CV% and bias% values were  
249 within 12% or lower for all the spiked analytes at both low and high concentrations.

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### 251 *Carry-over*

252 The background chromatographic profiles of the main transitions for each analyte, monitored during the  
253 analysis of blank hair extracts injected after samples spiked at the highest analytes concentration, did not

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3 254 show the presence of any significant signal (i.e. the S/N value was always <3) at the retention times  
4 255 expected for most of the tested analytes, with few exceptions. For dicoumarol and flocoumafen, S/N>3  
5 256 (S/N = 3.4 ÷ 8.9) was observed in all the replicates for both SRM transitions considered. We also evaluated  
6 257 the carry-over effect after a slightly lower spiking of hair samples, i.e. 250 µg/g. Measurable carry-over was  
7 258 recorded for dicoumarol and flocoumafen also in this case, and the evaluation criteria for specificity were  
8 259 respected. For acenocoumarol, S/N>3 was observed in two replicates out of five for both SRM transition  
9 260 considered. In conclusion, possible occurrence of carry-over effects have to be taken into account for these  
10 261 three analytes when they are detected at relatively high concentrations.  
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### 17 263 *Matrix effect*

18 264 The effect of the real hair matrix components appeared to be significant for some of the analytes tested  
19 265 (see Table 2). The results of 'absolute' matrix effect show an increasing ion suppression in the last part of  
20 266 the chromatographic run, when methanol exceeds 80% in the eluent mixture and most of the hydrophobic  
21 267 matrix components are likely to be eluted. This effect is probably due to the simple sample preparation  
22 268 procedure, which is effective and rapid but do not include a purification step. Notably, a contribution to the  
23 269 observed matrix effect from the addition of multiple spiking has to be excluded, because no co-elution  
24 270 among the analytes is observed. The IS-corrected values of 'relative' matrix effect show a significant  
25 271 improvement for warfarin and dicoumarol, whereas the large ion suppression observed for flocoumafen  
26 272 and brodifacoum take minimal advantage from the IS-correction. In real forensic cases, the quantitative  
27 273 results for these two analytes should be considered thoughtfully. Fully reliable results may be obtained by  
28 274 the standard addition quantification method, whenever high accuracy is needed. On the other hand, the  
29 275 good linearity observed in the calibration plots for these analytes demonstrated that the observed matrix  
30 276 effect is proportionally constant, i.e. does not depend on the analytes' concentrations.  
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### 41 278 **Application to a real case**

42 279 ~~A 97-years old woman was hospitalized because of severe symptoms, including drowsiness, convulsions,~~  
43 280 ~~pallor and hematoma. The laboratory tests showed abnormal values for coagulation parameters~~  
44 281 ~~(prothrombin time-international normalized ratio PT-INR = 12.46; activated partial thromboplastin time~~  
45 282 ~~aPTT = 60 s; aPTT ratio = 1.82). After intra-venous (IV) administration of 10 mg vitamin K and one day~~  
46 283 ~~monitoring, the patient recovered from the hemorrhagic syndrome (PT-INR = 1.45; aPTT = 40.6 s; aPTT ratio~~  
47 284 ~~= 1.23) and was dismissed. After one week, the woman was taken again to the Emergency Department (ED)~~  
48 285 ~~because she accused the same symptoms. Her coagulations parameters were the following: PT-INR = 13.31;~~  
49 286 ~~aPTT = 68 s; aPTT ratio = 2.06. After IV administration of vitamin K and three blood transfusions, the patient~~  
50 287 ~~recovered once more.~~  
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3 288 ~~During the treatment of the second episode, a blood sample was collected and screened for anticoagulants,~~  
4 289 ~~in order to find possible explanations. Screening for further substances, which may account for convulsive~~  
5 ~~symptoms, were not executed. Since the blood sample resulted positive to difenacoum, the case was~~  
6 ~~reported to the Public Prosecutor's office, which took jurisdiction of the case. A fruit mousse allegedly used~~  
7 291 ~~to poison the victim was seized by the Police. Our laboratory was asked by the Prosecutor to determine the~~  
8 292 ~~content of the mousse and to estimate for how long the poisoning occurred. In order to respond to the~~  
9 293 ~~latter query, the victim was asked to give a hair sample on which to execute the inherent toxicological~~  
10 294 ~~analyses. The patient's hair sample was taken 2.5 months after her first hospitalization.~~  
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16 296 The extraction of the fruit mousse was performed by QuEChERS method, which is a streamlined and  
17 297 effective extraction and cleanup approach for the analysis of a variety of analyte residues in food matrices  
18 298 ([3236](#)). The fruit mousse sample tested positive for difenacoum and  $\alpha$ -chloralose, at 2  $\mu\text{g/g}$  and 50  $\mu\text{g/g}$   
19 299 concentrations, respectively.  
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22 300 To ~~execute-perform~~ segmental analysis, proximal and distal extremities of the hair sample were identified.  
23 301 Assuming that the hair growth rate generally ranges from 1.0 to 1.3 cm/month ([3337](#)), a relationship  
24 302 between hair length and investigation chronology was obtained. The hair length was about 9 cm and was  
25 303 segmented as described in Table 4.  
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28 304 Difenacoum was detected in the first (proximal) 3-cm hair segment at the concentration of 2.9 pg/mg. To  
29 305 our knowledge, this is the first study to report that exposure to difenacoum is detectable in real hair  
30 306 samples. For most drugs, concentrations in the low picogram per milligram range are expected in the  
31 307 circumstances of single intake, such as in drug-facilitated crimes and drug offences ([2528](#), [348](#), [359](#)).  
32 308 Differently, long-term intoxication usually lead to nanogram of drug per milligram of hair levels ([3640](#),  
33 309 [3741](#)). Therefore, we concluded that in the present case the victim was administered difenacoum in either  
34 310 a single or few isolated occurrences, possibly immediately before the two admissions into the ED.  
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40 311 The other target analyte found in the hair sample was  $\alpha$ -chloralose, which was detected in the proximal (0-  
41 312 3 cm) segment at the concentration of 85 pg/mg. The two subsequent and consecutive segments (3-6 cm  
42 313 and 6-9 cm) showed only traces of difenacoum (below LOQ) and low but quantifiable levels of  $\alpha$ -chloralose  
43 314 (29 pg/mg and 6 pg/mg, respectively). Sporkert et al. reported a case of segmental hair analysis which  
44 315 yielded  $\alpha$ -chloralose concentrations in the range from 75 to 338 ng/mg for each segment, suggesting  
45 316 repetitive exposure of the victim to this substance ([3842](#)).  
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50 317 On the other hand, numerous factors may account for an observed longitudinal migration of drugs along  
51 318 the hair shaft ([2831](#)), suggesting that the detection of a drug in two or three hair segments does not  
52 319 necessarily implies multiple exposures. For example, drugs released in the sweat are prevalently  
53 320 incorporated into the proximal hair segment, but partly also in distal segments, especially when the entire  
54 321 hair length is kept in contact with the skull by a pillow, a foulard, a hat, or similar clothes. This is even more  
55 322 likely in elderly people spending most part of the day in armchairs and bed, as in the present case. In order  
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3 323 | to interpret apparently contradictory segmental hair analysis data after single drug exposure, Kintz ([3943](#))  
4 324 | proposed to consider that the highest concentration must be detected in the segment corresponding to the  
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6 325 | period of the alleged event, and this measured concentration should be at least three times higher than  
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8 326 | those measured in the preceding or following segments. In the case presented hereby, we concluded that  
9  
10 327 | the victim was repeatedly exposed to  $\alpha$ -chloralose in the period corresponding to the first segment of hair.  
11 328 | Possible contamination of the remaining hair segments may be accounted for by the fact that the victim  
12  
13 329 | used to spend most of her time in bed or on armchairs.

14 330 | Several harmful substances are easily available on the market in large quantities. Therefore, these  
15 331 | compounds are often involved in intoxication cases and detected in biological specimens, including hair, in  
16 332 | circumstances of attempted or accomplished poisonings. Cumulative exposure to organophosphorus  
17 333 | pesticides was demonstrated by Kavvalakis et al. 2012 (44), who reported results on hair samples from both  
18 334 | the general population and exposed populations. Concentrations of non-specific metabolites of  
19 335 | organophosphorus pesticides, dialkylphosphates, ranged from 40 to 165 ppb for the general population and  
20 336 | from 181.7 and 812.9 ppb for the exposed population. Similar hair concentrations were reported also by  
21 337 | Tsatsakis et al (45).

22 338 | Kavvalakis et al. 2013 demonstrated a dose dependent accumulation of Imidacloprid, a relatively new  
23 339 | neuro-active neonicotinoid insecticide, in rabbit hair, after a chronic sub-acute long term exposure to the  
24 340 | insecticide (46), while Schummer et al measured 50 pesticides including 39 molecules from different  
25 341 | chemical families currently used in agriculture and 11 organochlorines in hair of farm workers in order to  
26 342 | evaluate the exposure to pesticides (47). These results demonstrate that hair analysis can provide extensive  
27 343 | information on human exposure to pesticides and harmful substances in general.

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## 29 345 | **CONCLUSIONS**

30 346 | An UHPLC-MS/MS method for the simultaneous determination of ten anticoagulant rodenticides and  $\alpha$ -  
31 347 | chloralose in human hair was developed and validated. The method proved to be simple, accurate, rapid  
32 348 | and highly sensitive, allowing the simultaneous detection of all compounds. The method was successfully  
33 349 | applied to a real case of difenacoum and  $\alpha$ -chloralose poisoning and proved sensitive enough to detect  
34 350 | occasional exposure of the victim to the two analytes by segmental analysis.

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## REFERENCES

- 1  
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3 352  
4 353  
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6 354  
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8 355 1. Rady, G.H., El-Mahrouky, F.S., Abdelnabby, H.M., Ahmed, H.A. (2013) Sub-Lethal and Teratogenicity  
9 356 Action of Bromadiolone and Chlorophacinone Anticoagulant Rodenticides on Albino Rats.  
10 357 *American-Eurasian Journal of Toxicological Sciences*, **5**, 07-14.
- 11  
12 358 2. Jin, M., Ren, Y., Xu, X., Chen, X. (2007) Determination of bromadiolone in whole blood by high-  
13 359 performance liquid chromatography coupled with electrospray ionization tandem mass  
14 360 spectrometry. *Forensic Science International*, **171**, 52–56.
- 15  
16 361 3. Vandebroucke, V., Desmet, N., De Backer, P., Croubels, S. (2008) Multi-residue analysis of eight  
17 362 anticoagulant rodenticides in animal plasma and liver using liquid chromatography combined with  
18 363 heated electrospray ionization tandem mass spectrometry. *Journal of Chromatography B*, **869**,  
19 364 101–110.
- 20 365 4. Fourel, I., Hugnet, C., Goy-Thollot, I., Berny, P. (2012) Validation of a new liquid chromatography–  
21 366 tandem mass spectrometry ion-trap technique for the simultaneous determination of thirteen  
22 367 anticoagulant rodenticides, drugs, or natural products. *Journal of Analytical Toxicology*, **34**, 95-102.
- 23 368 5. Palmer, R.B., Alakija, P., Cde Baca, J.E., Nolte, K.B. (1999) Fatal Brodifacoum Rodenticide Poisoning:  
24 369 Autopsy and Toxicologic Findings. *Journal of Forensic Sciences*, **44**, 851-855.
- 25 370 6. Grobosch, T., Angelow, B., Schonberg, L., Tampe, D. (2006) Acute Bromadiolone Intoxication.  
26 371 *Journal of Analytical Toxicology*, **30**, 281-286.
- 27 372 4-7. King, N., Tran, M. (2015) Long-Acting Anticoagulant Rodenticide (Superwarfarin) Poisoning: A  
28 373 Review of Its Historical Development, Epidemiology, and Clinical Management. *Transfusion*  
29 374 *Medicine Reviews*, **29**, 250-258.
- 30 375 5-8. Bullard, R.W., Holguin, G., Peterson, J.E. (1975) Determination of chlorphacinone and diphenadione  
31 376 residues in biological materials. *Journal of Agricultural and Food Chemistry*, **23**, 72-74.
- 32 377 6-9. Duffield, P., Duffield, A.M., Kennedy, M., Birkett, D.J., Wade, D.N. (1979) Warfarin and warfarin-  
33 378 alcohol levels in anti-coagulated patients. *Australian & New Zealand Journal of Medicine*, **9**, 534-  
34 379 537.
- 35 380 7-10. \_\_\_\_\_ Mount, M.E., Kurth, M.J., Jackson, D.Y. (1988) Production of antibodies and development of  
36 381 an immunoassay for the anticoagulant, diphacinone. *Journal of Immunoassay*, **9**, 69-81.
- 37 382 8-11. \_\_\_\_\_ Berny, P.J., Buronfosse, T., Lorgue, G. (1995) Anticoagulant poisoning in animals: A simple  
38 383 new high-performance thin-layer chromatographic (HPTLC) method for the simultaneous  
39 384 determination of 8 anticoagulant rodenticides in liver samples. *Journal of Analytical Toxicology*, **19**,  
40 385 576-580.

- 1  
2  
3 386 | ~~9-12.~~ Palazoglu, M.G., Tor, E.R., Holstege, D.M., Galey, F.D. (1998) Multiresidue analysis of nine  
4 387 | anticoagulant rodenticides in serum. *Journal of Agricultural and Food Chemistry*, **46**, 4260-4266.  
5  
6 388 | ~~10-13.~~ Guan, F., Ishii, A., Seno, H., Watanabe, K., Kumazawa, T., Suzuki, O. (1999) A method for  
7 389 | simultaneous determination of five anticoagulant rodenticides in whole blood by high-performance  
8 390 | liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, **21**, 179-185.  
9  
10 391 | ~~11-14.~~ Kollroser, M., Schober, C. (2002) Determination of Coumarin-type Anticoagulants in Human  
11 392 | Plasma by HPLC–Electrospray Ionization Tandem Mass Spectrometry with an Ion Trap Detector.  
12 393 | *Clinical Chemistry*, **48**, 84–91.  
13  
14 394 | ~~12-15.~~ Jin, M., OuYang, X., Chen, X. (2007) High-performance liquid chromatography coupled with  
15 395 | electrospray ionization tandem mass spectrometry for the determination of flocoumafen and  
16 396 | brodifacoum in whole blood. *Journal of Applied Toxicology*, **27**, 18–24.  
17  
18 397 | ~~13-16.~~ Jin, M., Cai, M., Chen, X. (2009) Simultaneous Measurement of Indandione-Type  
19 398 | Rodenticides in Human Serum by Liquid Chromatography–Electrospray Ionization-Tandem Mass  
20 399 | Spectrometry. *Journal of Analytical Toxicology*, **33**, 294-300.  
21  
22 400 | ~~14-17.~~ Yan, H., Xiang, P., Zhu, L., Shen, M. (2012) Determination of bromadiolone and brodifacoum  
23 401 | in human blood using LC-ESI/MS/MS and its application in four superwarfarin poisoning cases.  
24 402 | *Forensic Science International*, **222**, 313–317.  
25  
26 403 | ~~15-18.~~ Gallochio, F., Basilicata, L., Benetti, C., Angeletti, R., Binato, G. (2014) Multi-residue  
27 404 | determination of eleven anticoagulant rodenticides by high-performance liquid chromatography  
28 405 | with diode array/fluorimetric detection: Investigation of suspected animal poisoning in the period  
29 406 | 2012–2013 in north-eastern Italy. *Forensic Science International*, **244**, 63-69.  
30  
31 407 | ~~16-19.~~ Bidny, S., Gago, K., David, M., Duong, T., Albertyn, D., Gunja, N. (2015) A Validated LC–MS–  
32 408 | MS Method for Simultaneous Identification and Quantitation of Rodenticides in Blood. *Journal of*  
33 409 | *Analytical Toxicology*, **39**, 219–224.  
34  
35 410 | ~~17-20.~~ Leikin, J.B., Paloucek, F.P. (2007) Poisoning and toxicology handbook, 4th edition. Informa  
36 411 | Healthcare, New York, NY.  
37  
38 412 | ~~18-21.~~ Federici, S., Claudet, I., Laporte-Turpin, E., Marcoux, M.O., Cheuret, E., Maréchal, K. (2006)  
39 413 | Intoxication sévère par le chloralose chez un nourrisson. *Archives of Pediatrics*, **13**, 364–366.  
40  
41 414 | ~~19-22.~~ Kouraichi, N., Brahmi, N., Elghord, H., Béji, O., Thabet, H., Amamou, M. (2010) Intoxication  
42 415 | par le chloralose: Facteurs pronostiques et prise en charge. *Reanimation*, **19**, 581–586.  
43  
44 416 | ~~20-23.~~ Kintz, P., Doray, S., Cirimele, V., Ludes, B. (1999) Testing for alpha-chloralose by headspace-  
45 417 | GC/MS. A case report. *Forensic Science International*, **104**, 55–63.  
46  
47 418 | ~~21-24.~~ Baselt, R.C. (2004) Disposition of toxic drugs and chemicals in man, 7th edition. Biomedical  
48 419 | Publications, Foster City, CA.

- 1  
2  
3 420 | ~~22-25.~~ Gerace, E., Ciccotelli, V., Rapetti, P., Salomone, A., Vincenti, M. (2012) Distribution of  
4 421 | Chloralose in a fatal intoxication. *Journal of Analytical Toxicology*, **36**, 452-456.  
5  
6 422 | ~~23-26.~~ Imran, M., Shafi, H., Wattoo, S.A., Chaudhary, M.T., Usman, H.F. (2015) Analytical methods  
7 423 | for determination of anticoagulant rodenticides in biological samples. *Forensic Science*  
8 424 | *International*, **253**, 94–102.  
9  
10 425 | ~~24-27.~~ Zhu, L., Yan, H., Shen, B., Shi, Y., Shen, M., Xiang, P. (2013) Determination of bromadiolone  
11 426 | and brodifacoum in human hair by liquid chromatography/tandem mass spectrometry and its  
12 427 | application to poisoning cases. *Rapid Communication in Mass Spectrometry*, **27**, 513–520.  
13  
14 428 | ~~25-28.~~ Kintz, P. (2012) Value of the concept of minimal detectable dosage in human hair. *Forensic*  
15 429 | *Science International*, **218**, 28–30.  
16  
17 430 | ~~26-29.~~ Vincenti, M., Salomone, A., Gerace, E., Pirro, V. (2013) Application of mass spectrometry to  
18 431 | hair analysis for forensic toxicological investigations. *Mass Spectrometry Reviews*, **32**, 312-332.  
19  
20 432 | ~~27-30.~~ Vincenti, M., Salomone, A., Gerace, E., Pirro, V. (2013) Role of LC-MS/MS in hair testing for  
21 433 | the determination of common drugs of abuse and other psychoactive drugs. *Bioanalysis*, **5**, 1919-  
22 434 | 1938.  
23  
24 435 | **31.** Vincenti, M., Kintz, P., (2015) New challenges and perspectives in hair analysis. In Kintz, P.,  
25 436 | Salomone, A., Vincenti, M. (eds.) *Hair Analysis in Clinical and Forensic Toxicology*, Chapter 12.  
26 437 | Elsevier – Academic Press, San Diego, CA, USA, 337-368.  
27  
28 438 | ~~28-32.~~ [Di Corcia, D., D'Urso, F., Gerace, E., Salomone, A., Vincenti, M. \(2012\) Simultaneous](#)  
29 439 | [determination in hair of multiclass drugs of abuse \(including THC\), by ultra-high performance liquid](#)  
30 440 | [chromatography–tandem mass spectrometry, \*Journal of Chromatography B\*, \*\*899\*\*, 154–159.](#)  
31  
32 441 | ~~29-33.~~ Scientific Working Group for Forensic Toxicology (SWGTOX). (2013) *Standard practices for*  
33 442 | *method validation in forensic toxicology*. *Journal of Analytical Toxicology*, **37**, 452–474.  
34  
35 443 | ~~30-34.~~ Hubaux, A., Vos, G. (1970) Decision and Detection limits for linear calibration curves.  
36 444 | *Analytical Chemistry*, **42**, 8.  
37  
38 445 | ~~31-35.~~ Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M. (2003) Strategies for the assessment  
39 446 | of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*,  
40 447 | **75**, 3019.  
41  
42 448 | ~~32-36.~~ Wilkowska, A., Biziuk, M. (2011) Determination of pesticide residues in food matrices using  
43 449 | the QuEChERS methodology. *Food Chemistry*, **125**, 803–812.  
44  
45 450 | ~~33-37.~~ Pecoraro, V., Astore, I.P.L. (1990) Measurement of hair growth under physiological  
46 451 | conditions. *Hair and Hair Diseases*, 237-254.  
47  
48 452 | ~~34-38.~~ Kintz, P., Alvarez, J., Deveaux, M., Dumestre, V., Gaillard, Y., Gaulier, J. (2014) Conflicting  
49 453 | Hair Testing Results Can Have an Impact in Courts: Interpretation of Single Exposure to Zolpidem.  
50 454 | *Journal of Analytical Toxicology*, **38**, 304-305.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



- 1  
2  
3 455 | ~~35-39.~~ Salomone, A., Gerace, E., Di Corcia, D., Martra, G., Petrarulo, M., Vincenti, M. (2012) Hair  
4 456 | analysis of drugs involved in drug-facilitated sexual assault and detection of zolpidem in a  
5 457 | suspected case. *International Journal of Legal Medicine*, **126**, 451-459.
- 6  
7  
8 458 | ~~36-40.~~ Gerace, E., Salomone, A., Pellegrino, S., Vincenti, M. (2012) *Evidence of Haldol (halopadol)*  
9 459 | *long-term intoxication. Forensic Science International*, **215**, 121-123.
- 10  
11 460 | ~~37-41.~~ Cooper, G.A.A., Kronstrand, R., Kintz, P. (2012) Society of Hair Testing guidelines for drug  
12 461 | testing in hair. *Forensic Science International*, **218**, 20–24.
- 13  
14 462 | ~~38-42.~~ Sporkert, F., Augsburger, M., Brandt-Casadevall, C., Mangin, P. (2005) *Annales de*  
15 463 | *Toxicologie Analytique*, **17**, 127.
- 16  
17 464 | 43. Kintz, P. (2013) Issues about axial diffusion during segmental hair analysis. *Therapeutic Drug*  
18 465 | *Monitoring*, **35**, 408–410.
- 19  
20 466 | 44. Kawvalakis, M.P., Tsatsakis, A.M. (2012) The atlas of dialkylphosphates; assessment of cumulative  
21 467 | human organophosphorus pesticides' exposure. *Forensic Science International*, **218**, 111–122.
- 22  
23 468 | 45. Tsatsakis, A.M., Barbounis, M.G., Kavalakisa, M., Kokkinakis, M., Terzi, I., Tzatzarakis, M.N. (2010)  
24 469 | Determination of dialkyl phosphates in human hair for the biomonitoring of exposure to  
25 470 | organophosphate pesticides. *Journal of Chromatography B*, **878**, 1246–1252.
- 26  
27 471 | 46. Kawvalakis, M.P., Tzatzarakis, M.N., Theodoropoulou, E.P., Barbounis, E.G., Tsakalof, A.K., Tsatsakis  
28 472 | A.M. (2013) Development and application of LC–APCI–MS method for biomonitoring of animal and  
29 473 | human exposure to imidacloprid. *Chemosphere*, **93**, 2612–2620.
- 30  
31 474 | ~~39-47.~~ Schummer, C., Salquèbre, G., Briand, O., Millet, M., Appenzeller, B.M.R. (2012) *Toxicology*  
32 475 | *Letters*, **210**, 203– 210.
- 33  
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Table 1. SRM transitions and corresponding potentials for target compounds and internal standard detection.

Analyte	t <sub>R</sub> (min)	Precursor Ion	DP (V)	EP (V)	Target			Qualifier			
					Fragment	CE (V)	CXP (V)	Fragment	(qualifier/quantifier %)	CE (V)	CXP (V)
Coumafuryl	1.39	297.1	-95	-9	240.2	-28	-11	211.2	56 %	-42	-9
α-chloralose	1.95	307.0	-118	-5	161.0	-15	-7	189.2	50 %	-13	-8
Warfarin	2.56	307.1	-118	-5	161.1	-26	-14	250.2	60 %	-28	-10
Coumatetralyl	2.56	291.0	-162	-10	141.1	-36	-12	143.0	36 %	-55	-16
Acenocoumarol	2.63	352.0	-101	-12	265.2	-41	-9	145.0	95 %	-59	-13
Coumachlor	3.08	341.2	-82	-6	161.1	-28	-13	117.1	25 %	-50	-10
Dicoumarol	3.33	335.1	-72	-7	161.1	-21	-10	-	-	-	-
Bromadiolone	3.96	525.1	-88	-8	78.9	-114	-10	250.2	58 %	-46	-10
Difenacoum	4.06	443.1	-98	-9	135.0	-43	-8	293.4	71 %	-43	-8
Flocoumafen	4.25	541.2	-74	-9	161.0	-45	-15	289.2	46 %	-45	-12
Brodifacoum	4.33	521.1	-108	-8	78.9	-115	-14	135.0	55 %	-47	-14
Warfarin- d5 (IS)	2.52	312.2	-100	-10	161.2	-21	-22	-	-	-	-
DP: Declustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Cell Exit Potential											

Table 2. Range of calibration, linearity, LODs and LOQs values and matrix effect for all analytes.

Analyte	Linearity range (pg/mg)	Linearity (Adj R <sup>2</sup> )	LOD (pg/mg)	LOQ (pg/mg)	Matrix effect without IS correction <sup>a</sup> (n=5)		Matrix effect with IS correction <sup>b</sup> (n=5)	
					Mean (±%)	CV (%)	Mean (±%)	CV (%)
Coumafuryl	5-500	0.9999	0.6	1.2	- 21	4.4	- 8.0	5.2
α-chloralose	5-500	0.9990	1.6	3.2	- 11	9.8	+ 5.0	13
Warfarin	5-500	0.9933	0.3	0.6	- 14	9.5	+ 1.0	2.7
Coumatetralyl	5-500	0.9999	0.8	1.6	0	8.0	+ 17	8.3
Acenocoumarol	5-500	0.9926	1.7	3.5	- 1	8.7	+ 16	10
Coumachlor	5-500	0.9998	1.1	2.2	- 11	10	+ 4	7.3
Dicoumarol	5-500	0.9997	1.4	2.9	- 6	9.9	+ 10	7.7
Bromadiolone	5-500	0.9999	0.9	1.8	- 43	9.0	- 33	17
Difenacoum	5-500	0.9998	1.3	2.7	- 48	9.2	- 38	9.6
Flocoumafen	5-500	1.000	0.5	1.0	- 70	5.5	- 66	11
Brodifacoum	5-500	0.9934	2.6	5.1	- 77	6.8	- 72	13

LOD: limit of detection; LOQ: limit of quantitation; CV%: per cent variation coefficient

<sup>a</sup> Matrix effect was evaluated using five different sources of hair (25pg/mg for all analytes). Absolute peak areas are considered without IS correction

<sup>b</sup> Matrix effect was evaluated on the same sources of hair, but in this case the matrix effect was compensated by the use of the IS (Warfarin d5)

Table 3. Intra- and inter-day precision (CV%) and accuracy (bias %) for each analyte tested.

Analyte	Intra-day (n=10)				Inter-day (n=30)			
	Precision (CV%)		Accuracy (bias%)		Precision (CV%)		Accuracy (bias%)	
	5 pg/mg	250 pg/mg	5 pg/mg	250 pg/mg	5 pg/mg	250 pg/mg	5 pg/mg	250 pg/mg
Coumafuryl	6.1	4.5	+ 7.4	+ 2.2	11	9.1	+ 2.5	- 1.8
α-chloralose	5.8	7.3	- 6.4	- 4.0	8.4	5.8	- 4.0	- 2.5
Warfarin	5.2	7.7	+ 6.3	- 12	6.9	7.1	- 0.3	- 11
Coumatetralyl	3.7	3.9	- 7.3	- 3.3	10	5.2	- 4.7	- 4.3
Acenocoumarol	4.2	6.6	- 0.5	- 6.7	6.4	7.8	- 4.8	- 10
Coumachlor	6.1	8.5	+ 7.0	- 4.2	9.5	6.7	- 0.2	- 2.8
Dicoumarol	7.4	11	+ 3.8	+ 9.6	7.5	8.7	+ 9.7	+ 7.0
Bromadiolone	7.4	8.1	- 0.2	+ 2.9	9.4	9.1	- 4.3	+ 5.0
Difenacoum	8.8	5.7	- 2.6	- 5.6	11	11	- 2.0	+ 1.5
Flocoumafen	6.7	6.9	+ 5.6	+ 8.4	11	8.6	+ 6.5	+ 8.0
Brodifacoum	9.6	8.7	- 4.6	+ 3.6	12	12	+ 2.4	+ 4.1

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Table 4. Difenacoum and  $\alpha$ -chloralose determined in authentic hair samples

Hair segment (cm)	Concentration (pg/mg)	
	Difenacoum	$\alpha$ -chloralose
0-3	2.9	85
3-6	< LOQ	29
6-9	< LOQ	6

For Review Only

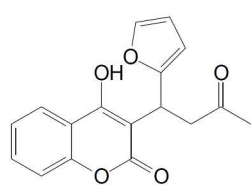
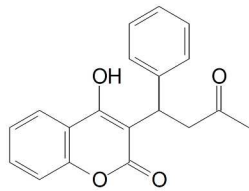
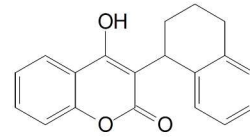
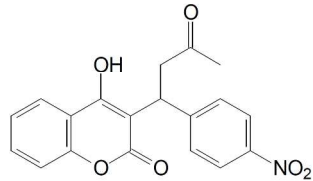
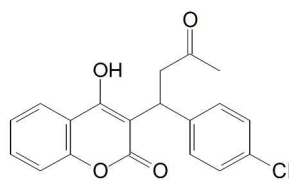
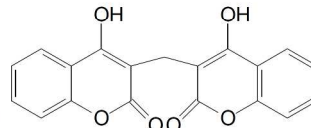
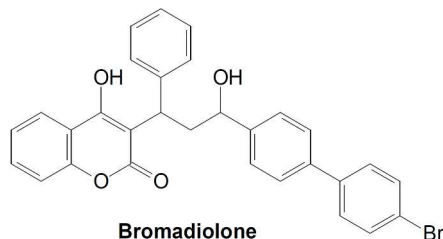
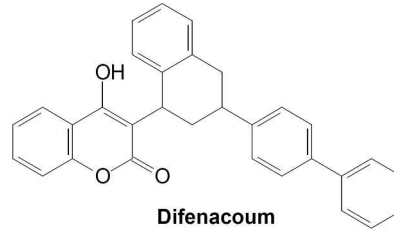
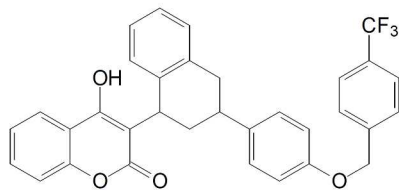
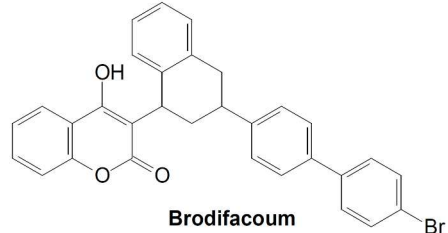
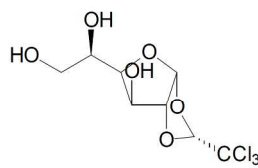
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3 **Figure captions**  
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7 Figure 1. Chemical structures of anticoagulant rodenticides and  $\alpha$ -chloralose considered in this study.  
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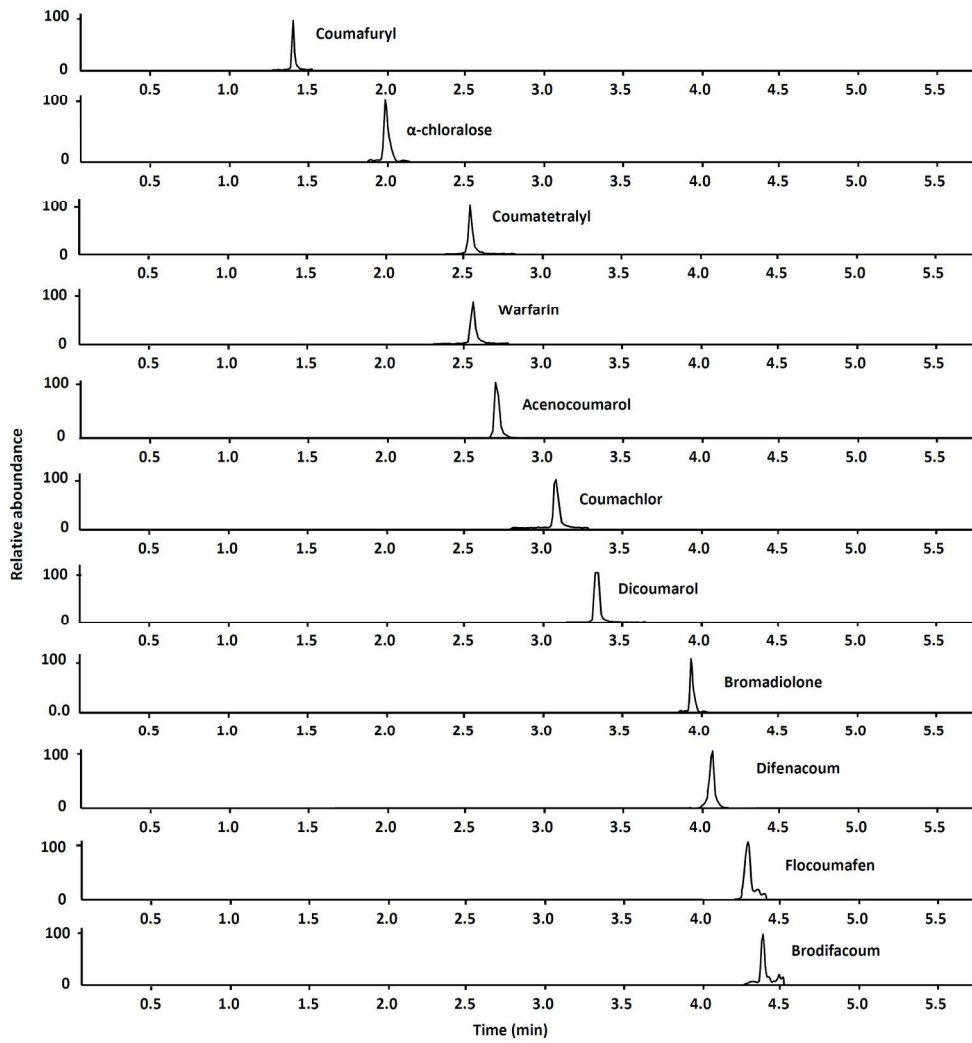
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11 Figure 2. SRM chromatograms of a blank hair sample spiked at 5  $\mu\text{g}/\text{mg}$   
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15 Figure 3. MRM chromatogram of the three segments of a real hair sample resulted positive to difenacoum  
16 (only segment A) and  $\alpha$ -chloralose.  
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For Review Only

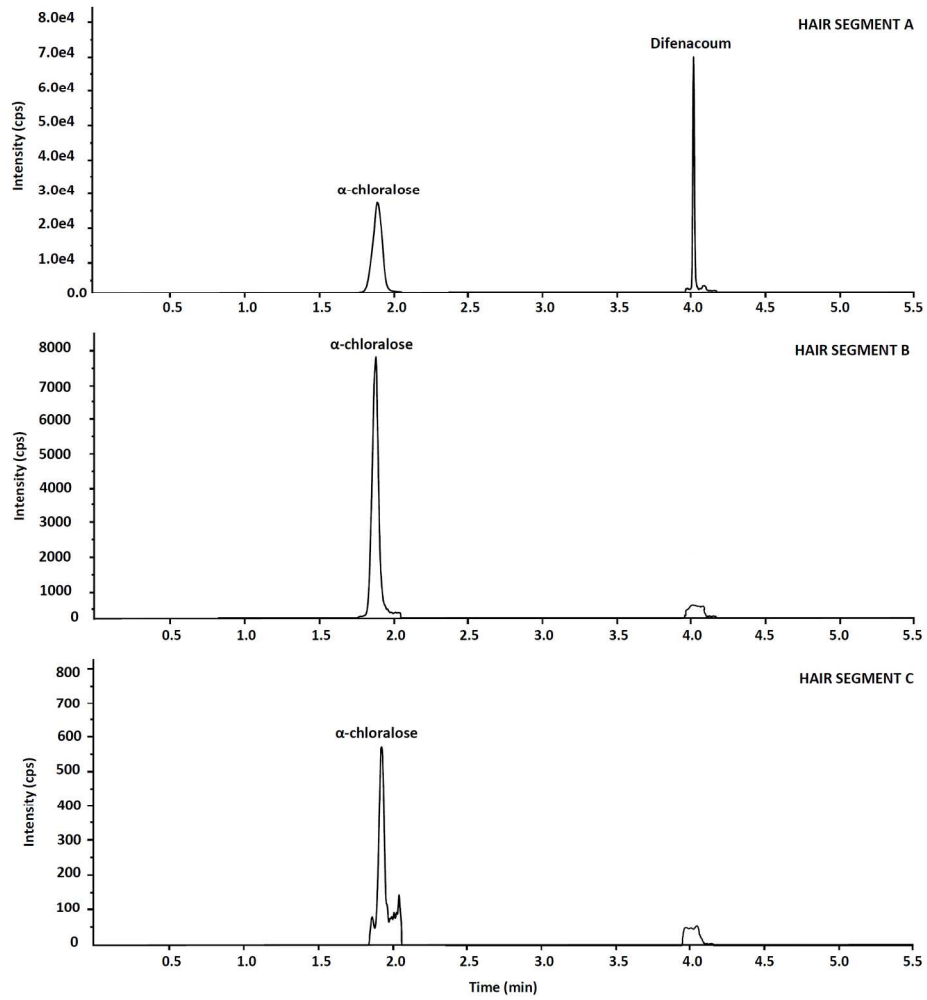
**Coumafuryl****Warfarin****Coumatetralyl****Acenocoumarol****Coumachlor****Dicumarol****Bromadiolone****Difenacoum****Flocoumafen****Brodifacoum** **$\alpha$ -chloralose**

154x190mm (600 x 600 DPI)



105x110mm (600 x 600 DPI)





101x103mm (600 x 600 DPI)



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