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DETERMINATION OF ANTICOAGULANT RODENTICIDES AND α-CHLORALOSE IN HUMAN HAIR 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 α-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 α-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 α-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 α-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.

KEYWORDS: UHPLC-MS/MS; hair; anticoagulant rodenticides; α-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 (~~5~~8), gas chromatography-mass spectrometry (~~6~~9), immunoassay (~~7~~10), thin-layer chromatography-UV/fluorescence (~~8~~11) and high-performance liquid chromatography (HPLC) with fluorescence and UV/photodiode array detectors (~~9-10~~12-13). In recent years, liquid chromatography coupled to mass spectrometry (LC-MS) has become the most frequently used technique for these analyses (~~11-16~~14-19). Besides anticoagulants, α-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 (~~17-19~~20-22). α-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 (~~20-22~~23-25). Many studies about rodenticides detection in biological samples were published in the past (~~23~~26), but only one on human hair, limited to brodifacoum and bromadiolone (~~24~~27).

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 f~~a~~urther periods corresponding to the hair segments

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more distant from the hair root (~~25-28~~28-31). Furthermore, hair analysis may provide a non-invasive approach for monitoring previous exposure also for wild animals, with easier storage and longer detection time compared to other non-invasive methods such as detection in pellets of bird of prey, or faeces of mammals (~~24~~27).

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

EXPERIMENTAL

~~Chemicals, reagents, and standard solutions~~Materials

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

~~Analytical method~~ Instrumental conditions

All analyses were performed on a Shimadzu Nexera 30 UHPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray Turbo Ion source operating in the negative (ESI–) ion mode. A Kinetex C18 column 50×2.1 mm i.d. × 1.7 µm (Phenomenex, Italy), protected by a C18 guard column, was used for the separation of analytes. The column oven was maintained at +40 °C, and the elution solvents used were water/ammonium formiate 2 mM (solvent A) and MeOH (solvent B). The mobile phase eluted under the following conditions (a/b; v/v): initial 80:20 ratio for 1 min, then linear gradient to 0:100 in 4 min; final 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, including the re-equilibration time at the initial conditions between two consecutive injections. The MS system was operated in the selected reaction monitoring mode (SRM). In order to establish appropriate SRM conditions, each analyte was individually infused into the ESI capillary, while the declustering potential (DP) and the entrance potential (EP) were adjusted to maximize the intensity of the $[M–H]^-$ species. The collision offset voltage (CE) was adjusted to preserve approximately 10 % of the precursor ion,

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and the cell exit potentials (CXP) were also optimized. Each SRM transition was maintained during a time window of ±10.0 s around the expected retention time of the corresponding analyte, and the SRM target scan time (i.e.,sum of dwell times for each SRM cycle) was 0.30 s, including pause times of 5 ms between consecutive SRM transitions. The best results were obtained using a source block temperature of +500 °C and an ion-spray voltage of -3.000 V. Both Q1 and Q3 were operated at unit mass resolution. Nitrogen was employed as the collision gas at 5×10^{-3} Pa. The gas settings were as follows: curtain gas 30.0 psi, collision 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) software was used for data processing. The chemical structures of the anticoagulant rodenticides investigated in this study and α-chloralose are reported in Figure 1. All analytes and IS, their corresponding retention time, SRM transitions, and potentials are presented in Table 1.

Case study

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

During the treatment of the second episode, a blood sample was collected and screened for anticoagulants, in order to find possible explanations. Screening for further substances, which may account for convulsive symptoms, were not performed. Since the blood sample resulted positive to difenacoum, the case was reported to the Public Prosecutor's office, which took jurisdiction of the case. A fruit mousse allegedly used to poison the victim was seized by the Police. Our laboratory was asked by the Prosecutor to determine the content of the mousse and to estimate for how long the poisoning occurred. In order to respond to the latter query, the victim was asked to give a hair sample on which to perform the inherent toxicological analyses. The patient's hair sample was taken 2.5 months after her first hospitalization.

Sample preparation

About 200 mg of hair was twice-washed with dichloromethane (2 mL, vortex mixed for 3 min). After complete removal of solvent wash, the hair was dried at room temperature by a gentle nitrogen flow and subsequently cut with scissors into 1–2 mm segments. An aliquot of about 50 mg was weighted and then fortified with 62.5 µL of warfarin-d5 ~~an IS~~ working solution at 20 ng/mL, yielding a final concentration of 25

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pg/mg. Similarly to the analysis of other xenobiotics in hair (32), ~~S~~sample extraction was carried out by addition of 1 mL of methanol, vortex shaking for 5 min and centrifuging at 4000 rpm for 3 min, to ensure the complete immersion of the matrix into the solvent, and final incubation at 55°C for 15 h. Lastly, the organic phase was collected, and evaporated to dryness under a gentle stream of nitrogen and mild heating (25°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in 100 µL of MeOH, transferred into a vial, centrifuged at 4000 rpm for 10 min. 2 µL of solution was injected into the UHPLC–MS/MS system.

Validation

The analytical method was validated in accordance with the criteria and recommendations of international standard and international guidelines (~~29~~33). The following parameters were investigated: specificity, selectivity, linearity range, detection and quantification limits (LOD and LOQ), intra-assay and inter-assay precision and accuracy. Carry-over and matrix effect were also investigated.

Specificity

A pool of five blank hair samples obtained from different healthy volunteers (two females, three males) was prepared without spiking and analyzed as described above. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM chromatograms characteristic for each investigated compound at the expected retention time interval. The S/N was measured on the less intense mass transition at the expected analyte retention time. The noise was measured from the end of the peak till ±0.05 min after it for each analyte. A S/N<3 was considered satisfactory in order to verify the method's specificity.

Selectivity

The repeatability of relative peak intensities for the transitions of each analyte was determined on five spiked hair samples at two concentration levels (5 and 250 pg/mg). Retention time precision at each concentration was also determined.

Linearity, LOD and LOQ

The linear calibration model was checked by analyzing (two replicates) blank hair samples spiked with the working solution at five concentration levels (5, 25, 100, 250, 500 pg/mg). The calibration was completed by internal standardization. The squared correlation coefficient, adjusted by taking into account the number of observations and independent variables (AdjR^2), was utilized to roughly estimate linearity.

The limit of detection (LOD) was estimated with the Hubaux-Vos approach (~~30~~34). LOQs was then 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 ±15% from the expected value.

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.

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%) (~~31~~35).

RESULTS AND DISCUSSION

Method development

The optimized UHPLC-MS/MS method allowed the simultaneous determination of 10 anticoagulant rodenticides plus α-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.

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

Validation

Specificity and Selectivity

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

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

Linearity, LOD and LOQ

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

Precision and accuracy

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

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

Carry-over

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

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

Matrix effect

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

Application to a real case

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

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~~During the treatment of the second episode, a blood sample was collected and screened for anticoagulants, in order to find possible explanations. Screening for further substances, which may account for convulsive symptoms, were not executed. Since the blood sample resulted positive to difenacoum, the case was reported to the Public Prosecutor's office, which took jurisdiction of the case. A fruit mousse allegedly used to poison the victim was seized by the Police. Our laboratory was asked by the Prosecutor to determine the content of the mousse and to estimate for how long the poisoning occurred. In order to respond to the latter query, the victim was asked to give a hair sample on which to execute the inherent toxicological analyses. The patient's hair sample was taken 2.5 months after her first hospitalization.~~

The extraction of the fruit mousse was performed by QuEChERS method, which is a streamlined and effective extraction and cleanup approach for the analysis of a variety of analyte residues in food matrices (~~32~~36). The fruit mousse sample tested positive for difenacoum and α-chloralose, at 2 µg/g and 50 µg/g concentrations, respectively.

To ~~execute~~ perform segmental analysis, proximal and distal extremities of the hair sample were identified. Assuming that the hair growth rate generally ranges from 1.0 to 1.3 cm/month (~~33~~37), a relationship between hair length and investigation chronology was obtained. The hair length was about 9 cm and was segmented as described in Table 4.

Difenacoum was detected in the first (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. For most drugs, concentrations in the low picogram per milligram range are expected in the circumstances of single intake, such as in drug-facilitated crimes and drug offences (~~25~~28, 3~~4~~8, 3~~5~~9). Differently, long-term intoxication usually lead to nanogram of drug per milligram of hair levels (~~36~~40, ~~37~~41). Therefore, we concluded that in the present case the victim was administered difenacoum in either a single or few isolated occurrences, possibly immediately before the two admissions into the ED.

The other target analyte found in the hair sample was α-chloralose, which was detected in the proximal (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 α-chloralose (29 pg/mg and 6 pg/mg, respectively). Sporkert et al. reported a case of segmental hair analysis which yielded α-chloralose concentrations in the range from 75 to 338 ng/mg for each segment, suggesting repetitive exposure of the victim to this substance (~~38~~42).

On the other hand, numerous factors may account for an observed longitudinal migration of drugs along the hair shaft (~~28~~31), suggesting that the detection of a drug in two or three hair segments does not necessarily implies multiple exposures. For example, drugs released in the sweat are prevalently incorporated into the proximal hair segment, but partly also in distal segments, especially when the entire hair length is kept in contact with the skull by a pillow, a foulard, a hat, or similar clothes. This is even more 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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to interpret apparently contradictory segmental hair analysis data after single drug exposure, Kintz (~~39~~43) proposed to consider that the highest concentration must be detected in the segment corresponding to the period of the alleged event, and this measured concentration should be at least three times higher than those measured in the preceding or following segments. In the case presented hereby, we concluded that the victim was repeatedly exposed to α-chloralose in the period corresponding to the first segment of hair. Possible contamination of the remaining hair segments may be accounted for by the fact that the victim used to spend most of her time in bed or on armchairs.

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

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

CONCLUSIONS

An UHPLC-MS/MS method for the simultaneous determination of ten anticoagulant rodenticides and α-chloralose in human hair was developed and validated. The method proved to be simple, accurate, rapid and highly sensitive, allowing the simultaneous detection of all compounds. The method was successfully applied to a real case of difenacoum and α-chloralose poisoning and proved sensitive enough to detect occasional exposure of the victim to the two analytes by segmental analysis.

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Table 1. SRM transitions and corresponding potentials for target compounds and internal standard detection.

					Target			Qualifier			
Analyte	**t_R (min)**	**Precursor Ion**	**DP (V)**	**EP (V)**	**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

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

					Matrix effect without IS correction[a] (n=5)		Matrix effect with IS correction[b] (n=5)	
Analyte	**Linearity range (pg/mg)**	**Linearity (Adj R^2)**	**LOD (pg/mg)**	**LOQ (pg/mg)**	**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

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

[b]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)

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

	Intra-day (n=10)					Inter-day (n=30)			
	Precision (CV%)		Accuracy (bias%)			Precision (CV%)		Accuracy (bias%)	
Analyte	**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 α-chloralose determined in authentic hair samples

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

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Figure captions

Figure 1. Chemical structures of anticoagulant rodenticides and α-chloralose considered in this study.

Figure 2. SRM chromatograms of a blank hair sample spiked at 5 pg/mg

Figure 3. MRM chromatogram of the three segments of a real hair sample resulted positive to difenacoum (only segment A) and α-chloralose.

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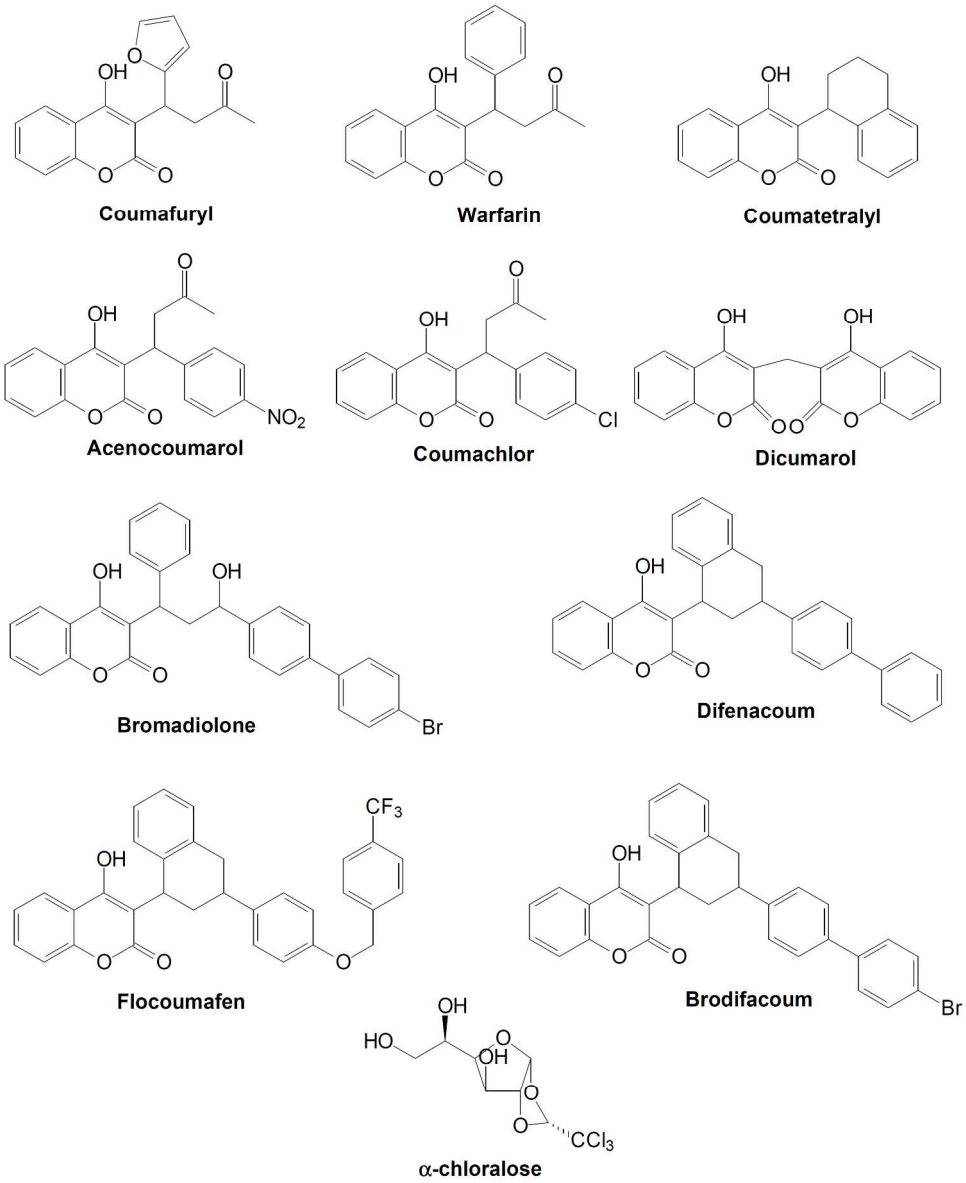

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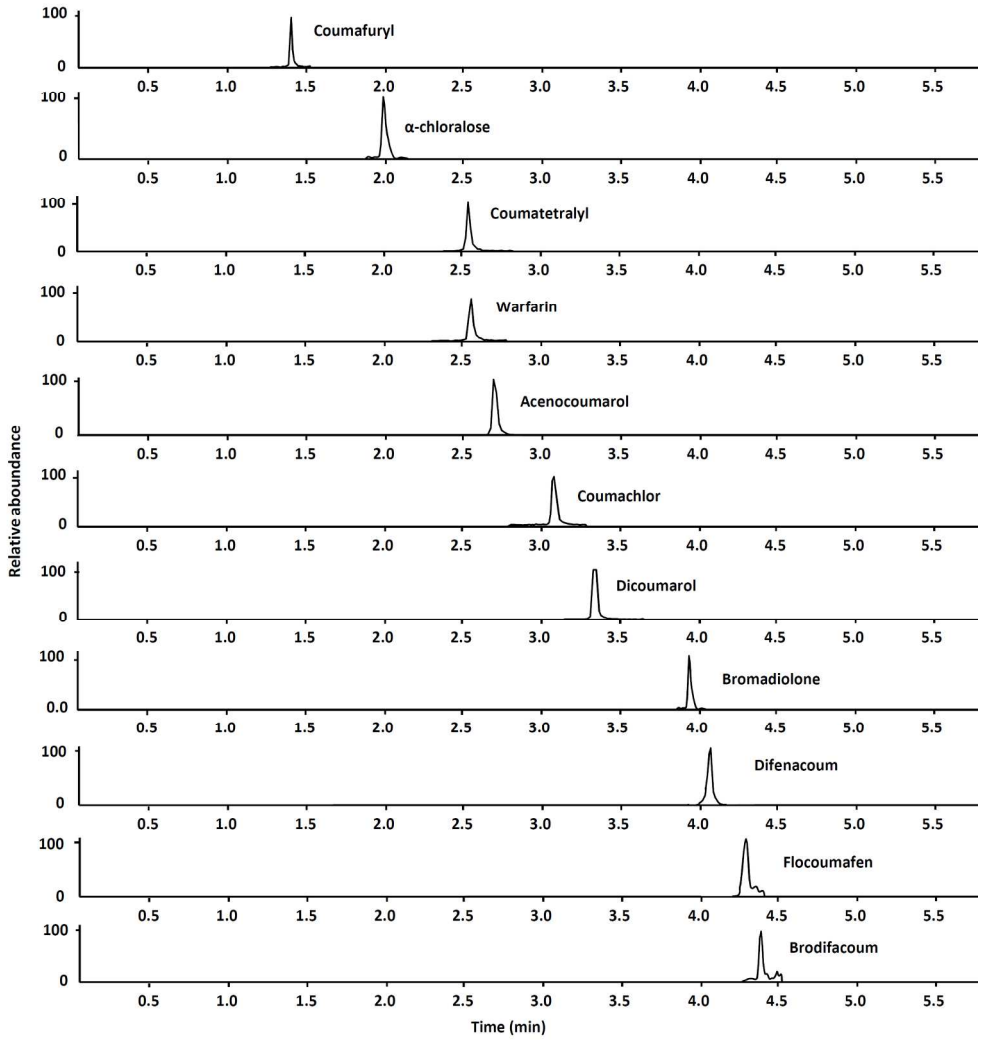

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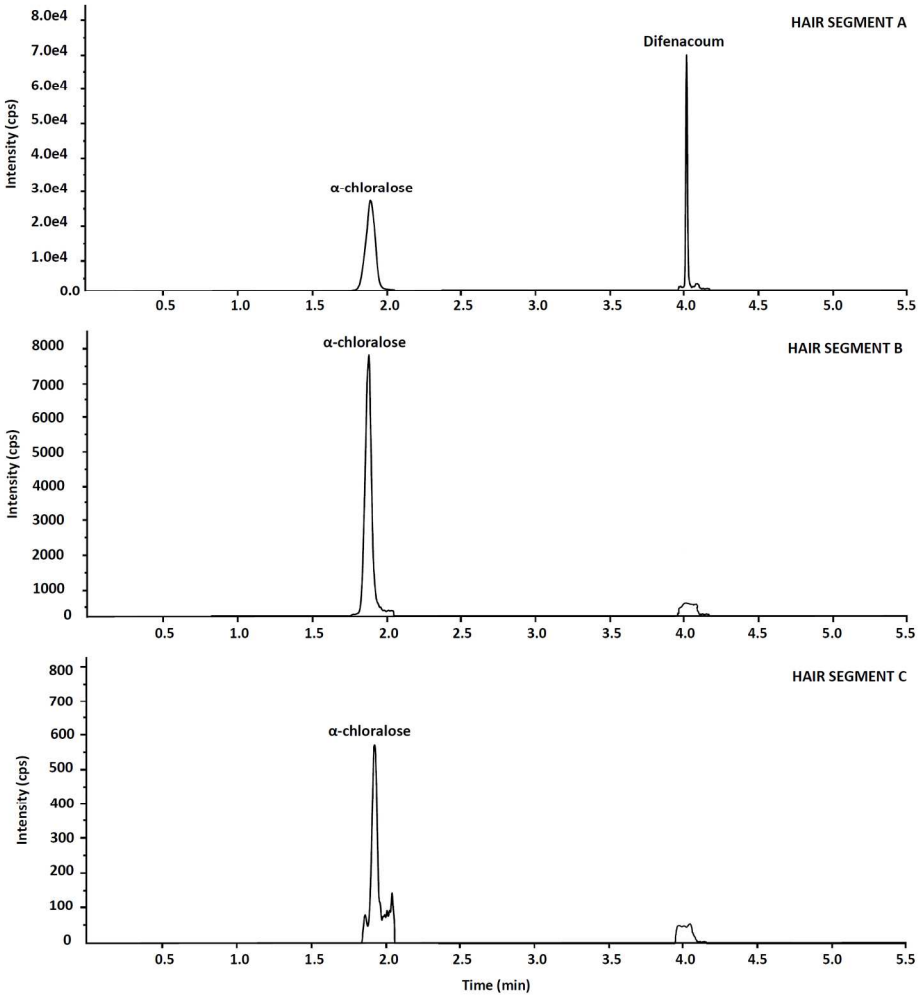

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