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Multiresidue Analysis of Seven Anticoagulant Rodenticides by High-Performance Liquid Chromatography/Electrospray/Mass Spectrometry

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Mice and rat populations are commonly controlled by two classes of rodenticide anticoagulants, coumarins and indandiones. However, poisoning of nontarget animals also often occurs. For cases such as these, a rapid, multiresidue method, which provides positive confirmation for both classes of anticoagulant rodenticides, is needed by diagnostic laboratories. A method was developed for the determination of seven anticoagulant rodenticides, coumafuryl, pindone, warfarin, diphacinone, chlorophacinone, bromadiolone, and brodifacoum, in diverse matrices, animal feed, cooked beef, and fruit-flavored beverages using high-performance liquid chromatography/electrospray/mass spectrometry. Detection was by MS/MS with electrospray ionization in negative mode. Confirmation was by retention time, *m/z* of molecular ion, and two parent−daughter transitions. Recoveries from selected the matrices ranged from 61 to 117%. Limits of quantitation were as low as 1.5−4.5 ng g^{-1}. The developed method was rapid and provided the simultaneous confirmation and quantification of the seven anticoagulant rodenticides.

KEYWORDS: LC/ESI/MS/MS; rodenticides; coumarins; indandiones; multiresidue analysis

INTRODUCTION

Mice and rat populations are commonly controlled by two classes of rodenticide anticoagulants, coumarins and indandiones. However, poisoning of nontarget animals also often occurs when the anticoagulant rodenticides are applied to feed stocks used to attract the rodents. Nontarget animals can be poisoned by ingesting either the treated feeds or the poisoned rodents (*1*). Humans are also poisoned by rodenticides, which were among the most frequently used chemicals in deliberate self-poisonings (*2*) and in childhood poisonings (*3*). In the past 3 years, the Minnesota Department of Agriculture has been asked to perform analyses of beverage samples and animal feed in connection to identification of sources in suspected poisoning cases involving anticoagulant pesticides in these matrices. In cases such as these, diagnostic laboratories need a rapid, multiresidue method, which provides positive confirmation for both classes of anticoagulant rodenticides.

At present, a number of techniques have been used to detect selected anticoagulant rodenticides, including gas chromatography−electron capture detection (*4*), gas chromatography−mass spectrometry (*5*), thin-layer chromatography−UV/fluorescence (*6−8*), and immunoassay (*9*). The most commonly used method is high-performance liquid chromatography (HPLC)

with UV or fluorescence detection for single chemical analyses. However, there are a few multianalyte methods for analysis of anticoagulant rodenticides by HPLC with fluorescence and UV/photodiode array detectors (*10−15*). All of the above methods use a combination of fluorescence and UV/photodiode array detectors for confirmation.

For improved detection and confirmation of nonvolatile analytes, including anticoagulant rodenticides, HPLCs have been interfaced to mass spectrometers. For instance, thermospray and particle beam liquid chromatographic mass spectrometry analyses of coumarin anticoagulants have been reported (*16*). HPLC coupled to a MS detector by an atmospheric pressure chemical ionization (APCI) interface using an ion-pairing reagent has been used for analysis of four anticoagulant rodenticides in blood and urine (*17*). More recently, liquid chromatography coupled to electrospray ionization mass spectrometry (LC/ESI/MS) has been used for detection and confirmation of two indandione rodenticides in rodent bait (*18*) and LC/turbo ionspray/MS/MS has been used for the detection and confirmation of coumarin rodenticides in animal tissues (*19*).

The objective of this study was to develop a LC/ESI/MS/MS method for the simultaneous determination of the most commonly used coumarin and indandione anticoagulant rodenticides (i.e., coumafuryl, warfarin, pindone, diphacinone, chlorophacinone, bromadiolone, and brodifacoum) in the matrices most commonly associated with the accidental or intentional misuse of these chemicals, such as in animal feed stocks or

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baits and liquid matrices. The developed LC/ESI/MS/MS method must be rapid, have minimal cleanup, and provide the simultaneous detection and confirmation of the seven antico-agulant rodenticides.

MATERIALS AND METHODS

Chemicals and Materials. Coumafuryl, {3-[1-(2-furanyl)-3-oxobu-tyl]-4-hydroxy-2*H*-1-benzopyran-2-one} (98% pure), pindone [2-(2,2-dimethyl-1-oxopropyl)-1*H*-indene-1,3(2*H*)-dione] (99.4%), warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one] (99.8%), diphacinone [2-(phenylacetyl)-1*H*-indene-1,3(2*H*)-dione] (99.3%), chlo-rophacinone {2-[(4-chlorophenyl)phenylacetyl]-1*H*-indene-1,3(2*H*)-di-one} (99.6%), bromadiolone {3-[3-(4′-bromo[1,1′-biphenyl]-4-yl)-3-hydroxy-1-phenylpropyl]-4-hydroxy-2*H*-1-benzopyran-2-one} (97%), and brodifacoum {3-[3-(4′-bromo[1-1′-biphenyl]-4-yl)-1,2,3,4-tetrahy-dro-1-napthalenyl]-4-hydroxy-2*H*-1-benzopyran-2-one} (98.9%) (struc-tures are in Figure 1) were used as received from the U.S. Environ-mental Protection Agency. Commercially available rat baits containing brodifacoum (0.005% active ingredient) and diphacinone (0.005% active ingredient) were purchased from a local hardware store.

Standard and Matrices Preparation. An initial standard of each rodenticide was made by weighing ~10 mg of analytical standard and then, using a burette, adding the exact volume of methanol needed to obtain a 1000 μg mL^{-1} (after calculating % purity) solution. A 5 μg mL^{-1} working standard mixture of all rodenticides was prepared by dilution with methanol. The working standard mixture was used as a spiking solution and was diluted with 70% 10 mM ammonium acetate: 30% methanol (v:v) to prepare a series of standard solutions for creation of a calibration curve from 0.2 to 50 ng mL^{-1}. An infusing solution for optimization of MS conditions of each rodenticide was prepared by adding a few drops of the 1000 μg mL^{-1} standard into ~3 mL of 30% 10 mM ammonium acetate:70% methanol (v:v) solution. The relatively high % methanol was to ensure dissolution of the chemicals in the infusing solution.

Samples (2 g) of commercially available soybean meal feed samples and cooked ground beef were treated with 60 μL of 5 μg mL^{-1} standard mixture containing all rodenticides and thoroughly mixed, and the solvent was allowed to evaporate. The final concentration of each of the seven chemicals was 0.15 μg g^{-1}. Ground shavings (10 mg) from commercial rat bait pellets containing brodifacoum were also added to 2 g of soybean meal feed samples, and ground shavings (10 mg) from commercial rat bait pellets containing brodifacoum and diphacinone were added to 2 g of cooked ground beef. The final concentrations of brodifacoum and diphacinone were 0.25 μg g^{-1}.

A powdered cherry-flavored drink mix was prepared according to package directions. A 1 mL aliquot of the 5 μg mL^{-1} standard mixture containing all rodenticides was placed in a 25 mL volumetric flask, and the flask was filled to the mark with drink mix, resulting in a final concentration of 0.2 μg mL^{-1} for each chemical. Ground commercial rat bait pellets containing brodificoum and diphacinone (1 g each) were placed in a 250 mL volumetric flask, the flask was filled to the mark with drink mix, and the flask was thoroughly mixed. To facilitate the dissolution of the rodenticides, the drink was heated at 50 °C for 1 h, as opposed to letting the solution sit at room temperature for a long time. Assuming that all of the active ingredients were dissolved, the final concentration of each chemical would be 0.2 μg mL^{-1} in the drink mix.

Extraction Procedure. Samples (2 g) of treated soybean meal feed or ground beef were placed into 50 mL polypropylene centrifuge tubes, along with 5 mL of water. The samples were agitated with a vortex mixer and then allowed to sit for 5 min. After 10 mL of acetonitrile was added, the sample was again agitated with a vortex mixer. After it was shaked for 30 min on a linear shaker, the sample was centrifuged at 3000 rpm (1540*g*) for 10 min.

A 2.0 mL aliquot was passed by gravity through a Varian Bond Elut Alumina Basic (ALB) solid-phase extraction (SPE) column (6 cm³/1 g), which had been conditioned sequentially with methanol, water, and acetonitrile, and the effluent was collected in 15 mL test tube. The ALB column was rinsed with 9 mL of acetonitrile, which was collected with the sample. The sample was transferred to a 40 mL

Table 1. LC/ESI/MS/MS Analysis Parameters for Seven Rodenticides

rodenticide	precursor	RT (min)	parent (Da)	daughter (Da)	cone (V)	collision (V)
coumafuryl	[M − H]$^-$	5.5	**296.9**^{a}	**161.3**	38	18
			296.9	240.0	38	20
pindone	[M − H]$^-$	10.9	**229.1**	**116.0**	46	36
			229.1	144.0	46	25
warfarin	[M − H]$^-$	11.2	**306.9**	**161.2**	38	19
			306.9	250.0	38	22
diphacinone	[M − H]$^-$	14.8	**338.9**	**167.1**	38	24
			338.9	115.9	38	45
chlorophacinone	[M − H]$^-$	17.0	**372.9**	**200.9**	45	22
			372.9	145.4	45	20
bromadiolone	[M − H]$^-$	18.2	**526.9**	**250.0**	60	34
			526.9	80.9	60	37
brodifacoum	[M − H]$^-$	20.2	**523.0**	**80.8**	59	38
			523.0	135.0	59	35

^{a} Transitions in bold were used for quantitation.

concentration tube, and 0.6 mL water was added. The solution was evaporated at 50 °C using a Turbovap to ~0.5 mL. Methanol was added to the 1.0 mL mark on the tube and mixed. After the solution was brought to a final volume of ~2 mL by adding 1.0 mL of 10 mM ammonium acetate, the sample was mixed with a vortex mixer and then sonicated for 3 min. The solution was passed through a 0.2 μm PTFE Acrodisc syringe filter into an autosampler vial for analysis.

An aliquot (0.2 mL) of drink mix treated with all seven chemicals was added to 2 mL of water. The chemicals were extracted from the diluted drink mix and from 2 mL of water treated with 8 μL of the 5 μg mL^{-1} standard mixture by four liquid−liquid extractions using dichloromethane (4:1 v:v aqueous:dichloromethane). The combined extracts were evaporated just to dryness. The chemicals were dissolved in 0.5 mL of methanol, and the solution was brought to a final volume of 2 mL with 10 mM ammonium acetate for analysis. For comparison, an aliquot (0.2 mL) of drink mix treated with all seven chemicals was mixed with 0.5 mL of methanol and brought to a final volume of 2.0 with 10 mM ammonium acetate for analysis.

The solutions in the flasks containing ground commercial bait in the drink mix were decanted, and 0.2 mL aliquots were mixed with 0.5 mL of methanol and brought to a final volume of 2.0 mL with 10 mM ammonium acetate for analysis. Aliquots (0.2 mL) were also extracted with dichloromethane as described above. A portion (60 mg) of the moist solid residue remaining in the flask was removed and placed in a centrifuge tube with 10 mL of acetonitrile. The sample was agitated with a vortex mixer, shaken for 30 min on a linear shaker, and centrifuged at 3000 rpm (1540*g*) for 10 min. An aliquot (1 mL) of the supernant was diluted to 5 mL with 1:4 (v/v) methanol:10 mM ammonium acetate for analysis.

LC/ESI/MS/MS. The LC/ESI/MS/MS system used was an Alliance HT 2795 HPLC coupled with a Micromass Quattro Micro tandem quadrupole mass spectrometer with an electrospray interface and MassLynx 4.0 software (Waters, Milford, MA). Separation was performed using a Zorbax RX-C8 (2.1 mm × 150 mm × 5 μm) column with a mobile phase gradient of 10 mM ammonium acetate and methanol. The mobile phase gradient elution was 70% 10 mM ammonium acetate:30% methanol (v:v) from 0 to 4 min, increasing to 50% methanol at 9 min, to 70% methanol at 14 min, and to 95% methanol at 18 min, with a total run time of 28 min. The injection volume was 5 μL. Detection was by MS/MS with electrospray ionization in negative mode.

On the basis of infusion experiments, optimized instrument condi-tions were as follows: capillary, 2.5 kV; extractor, 1.0 V; source temperature, 120 °C; desolvation temperature, 500 °C; desolvation gas flow, 700 L h^{-1}; and cone gas flow, 50 L h^{-1}. For each compound, cone and collision voltages, which are ion-dependent, are listed in **Table 1**.

RESULTS AND DISCUSSION

LC/MS and LC/MS/MS are increasingly being used to analyze nonvolatile, labile organic chemicals, such as pesticides,

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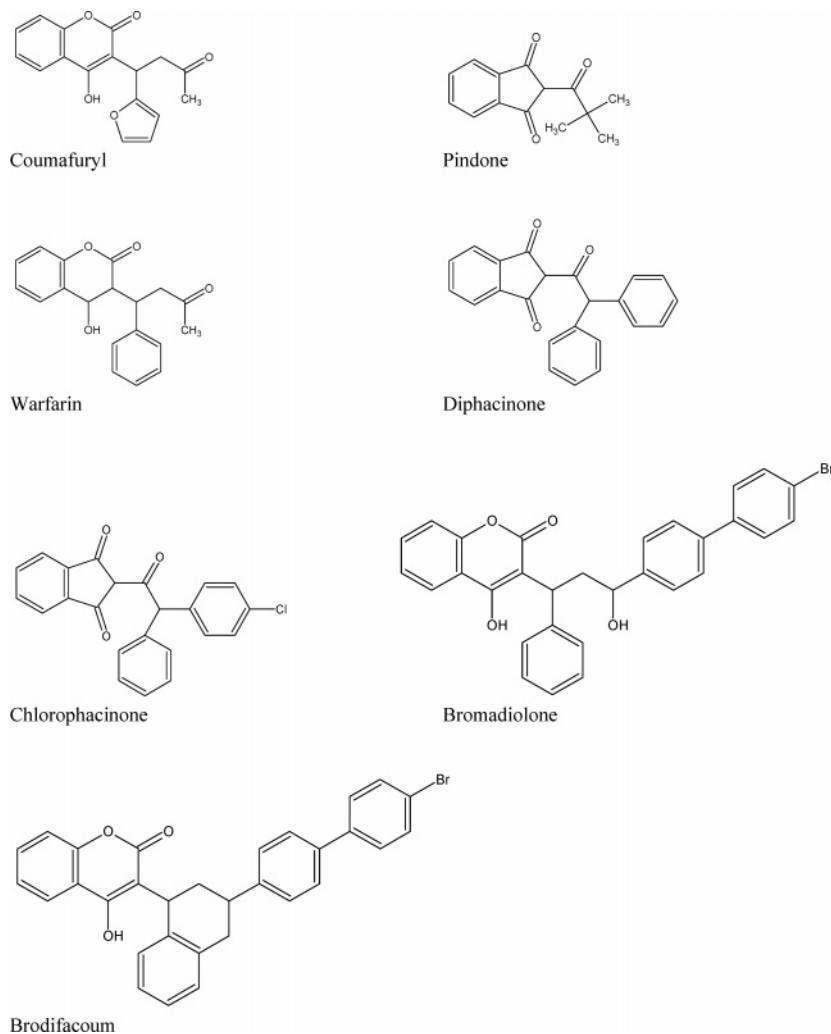
Figure 1. Chemical structures of seven anticoagulant rodenticides.

in soil, water, air, and food. If developed LC/MS/MS methods are to gain widespread acceptance, most importantly, they should provide confirmation of multiple analytes, but they should also be sufficiently sensitive to allow quantitation at trace levels, if necessary, in the desired matrix. The methods should also be rapid, with minimal cleanup. These criteria are particularly important in forensic cases, such as chemical poisoning.

The ESI mass spectra obtained while operating an LC/ESI/MS/MS in negative ion mode exhibited a $[M - H]^-$ signal and two parent−daughter transitions, which could be used for confirmation and quantitation (monitored quantitation transitions are listed in bold) (**Table 1**). The $[M - H]^-$ ion and at least one parent−daughter transition are consistent with those reported for diphacinone and chlorophacinone (*18*) and warfarin, bromadiolone, and brodifacoum (*19*). There are no previously published reports for coumafuryl and pindone. Although changing the pH of the infusing solution by addition of NH_4OH could increase the signals for the analytes, it was found that use of chromatographic mobile phase 70% 10 mM ammonium acetate: 30% methanol (v:v) also provided acceptable responses for confirmation and quantitation.

Both classes of anticoagulant rodenticides could be separated using a C8 column, with retention times ranging from 5.5 to 20.2 min, with a total run time of <29 min. **Figure 2** shows a LC/ESI/MS/MS chromatogram monitoring the quantitation transition of a 2 ng mL^{-1} standard solution containing all seven rodenticides. In other studies, an amine column provided

separation of two indandione rodenticides (*18*), while a phenyl-hexyl column provided separation of coumarin rodenticides (*19*). In our case, the C8 column could also separate the two diastereoisomers for bromadiolone.

Confirmation criteria subsequently used for the rodenticides were retention time match (±3%), *m/z* of molecular ion, and two parent−daughter transitions (see **Table 1**) and a signal-to-noise ratio (S/N) > 3. To obtain this level of confirmation, LC/ESI/MS/MS would be the method of choice; however, LC/ESI/MS may also work. For instance, although it was previously shown (*18*) that for diphacinone and chlorophacinone the full-scan LC/ESI/MS showed a very intense $[M - H]^-$, there was no fragmentation. In contrast, we were able to get a molecular ion, $[M - H]^-$, and the two fragments necessary for confirmation of diphacinone using a LC/ESI/MS (data not shown). In a LC/APCI/MS method, which required a di-*n*-butylammonium acetate ion-pairing reagent for analyses of five anticoagulant rodenticides, it was found that fragment ions that could be used for confirmation were not produced (*17*). Although ESI and APCI produced the same signal intensity, the authors used APCI because the hot vaporizer of the APCI interface was expected to breakdown the ion-pairing reagent.

Under the optimized MS/MS conditions, the calibration curve was linear for all seven chemicals from 0.2 to 20 ng mL^{-1}. Correlation coefficients, r^2, were >0.99 for six of the chemicals (0.991−0.998) and 0.984 for brodifacoum. It is recommended that the standard curve range not exceed 30 ng mL^{-1}. At 50 ng
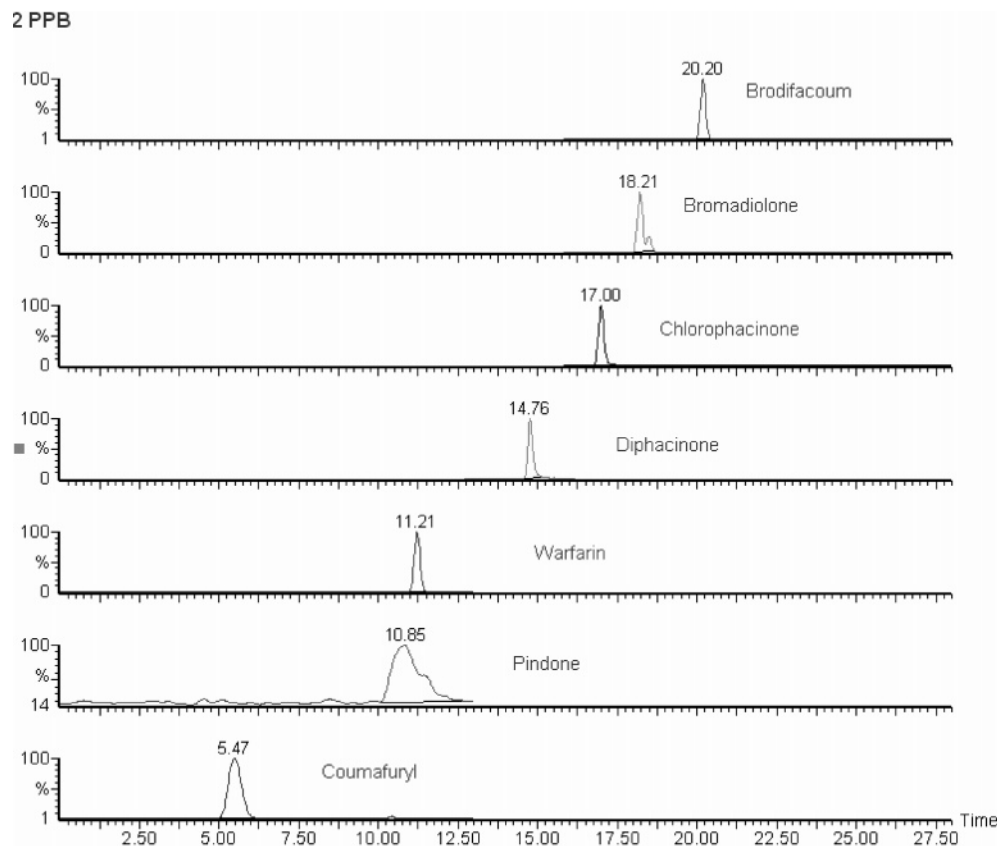
Figure 2. LC/ES/MS/MS chromatogram of a 2 ng mL^{-1} standard containing seven anticoagulant rodenticides.

mL^{-1}, the response was no longer linear and there were carryover problems with some of the rodenticides. To avoid carryover problems, multiple solvent rinses of the syringe, followed by multiple solvent injections, were necessary to remove the carried over chemicals.

In a screening method, it is difficult to have a range of standards in different matrices that would encompass all potential scenarios with which an analyst could be confronted. For instance, at the low end of the range, the method must be very sensitive to be able to rule out possible trace levels (i.e., <0.1 ng mL^{-1} in the vial) of the rodenticides if indeed they are not present. At the high end of the range, e.g., in the case of rat bait granules mixed with animal feeds, the granules can be seen and picked out of the feed for analyses. Even when 10 mg of rat bait shavings was mixed with 2 g of animal feed of ground beef, we could distinguish the formulated product from the feed. If the suspected particles were to be selectively removed, along with some of the feed, the residue concentrations would then only be considered semiquantitative but would still provide confirmation of the analyte.

Although recovery studies of all rodenticides from all matrices to be analyzed would be desirable, an analyst could never exactly reproduce the matrix that was received for analysis. However, it is necessary to show that the method is sufficiently rapid and robust and that it can encompass a variety of matrices. The developed method is relatively rapid. The total time for extraction and sample preparation for solid matrices, which included a SPE cleanup step, was <90 min. For some matrices, it may be possible to eliminate the SPE cleanup step. Although moisture affects the retention properties of alumina (*20*), it appeared to remove some possible interferences, while not affecting the rodenticides passing through the cartridge in acetonitrile. It should be noted that in preliminary method development experiments, some of the rodenticides could not

Table 2. Recoveries of Rodenticides from Animal Feed and Ground Beef Treated at 0.15 μg g^{-1} and from Drink Mix Treated at 0.20 μg mL^{-1}

rodenticide	% of applied^a feed recovery	% of applied^b beef recovery	drink recovery	LOQ (ng g^{-1})
coumafuryl	63 ± 5	88 ± 5	56	3.0
pindone	102 ± 2	80 ± 3	93 ± 3	4.5
warfarin	88 ± 1	78 ± 7	92 ± 9	1.5
diphacinone	107 ± 2	61 ± 4	81 ± 6	1.5
chlorophacinone	117 ± 4	65 ± 5	70 ± 6	1.5
bromadiolone	106 ± 6	117 ± 4	55 ± 7	3.0
brodifacoum	107 ± 15	109 ± 5	1 ± 1	3.0

^{a} Recovery mean ± standard deviation of five treated samples. ^{b} Recovery mean ± standard deviation of three treated samples.

be eluted from C18 SPE cartridges regardless of solvent polarity, resulting in very low recoveries.

The developed screening method, although semiquantitative as the result of the measured solvent volumes in the extraction step, was sufficiently sensitive for analysis of the seven rodenticides in animal feed and ground beef. Recovery of the six of the seven rodenticides from animal feed treated at 150 ng g^{-1} was quantitative (88−117% of applied) (**Table 2**). Recovery of coumafuryl from animal feed was 63%; while somewhat low, it is still acceptable for screening for the presence of the chemical. Recoveries of the rodenticides from ground beef were similar, 61−110%. The estimated limit of quantitation (LOQ) for the rodenticides in both matrices was <5 ng g^{-1} (**Table 2**). The LOQs were determined using the parent−daughter transition (**Table 1**) that provided the most intense signal. The LOQs were estimated from the lowest fortification levels of the rodenticides with a measured S/N > 10.

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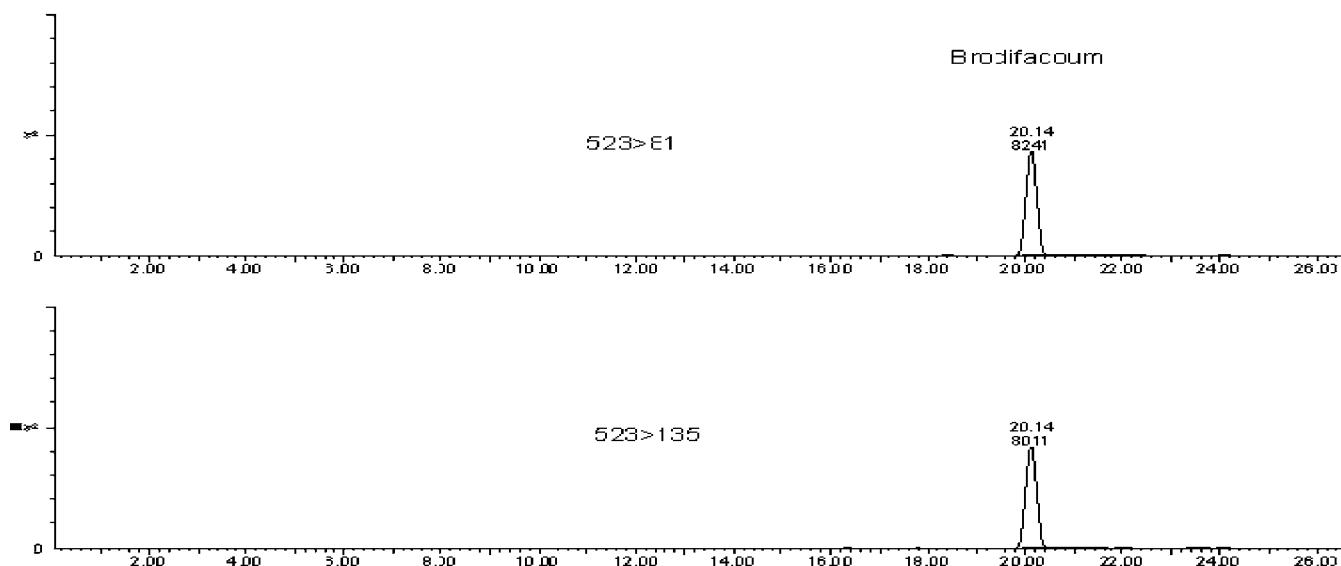*J. Agric. Food Chem.,* Vol. 55, No. 3, 2007 **575**

BAITCHECK

Figure 3. Chromatogram of an extract of animal feed treated with d-CON rat pellets (brodifacoum active ingredient).

Acceptable recoveries from prepared drink mix were obtained for five of the seven rodenticides (**Table 2**) either by direct analysis or after liquid−liquid extraction. Recovery of coumafuryl by direct analysis was >200% as the result of ion enhancement, which could be eliminated using liquid−liquid extraction (56% recovery). It appears that brodifacoum was not soluble in the drink mix, presumably due to the amount of dissolved sugar in the prepared drink. When brodificoum was added to distilled water, the recovery using liquid−liquid extraction was 54%.

In poisoning cases, formulated rodenticides, as opposed to analytical grade chemicals, would be mixed with a given matrix. **Figure 3** shows a chromatogram of an extract of animal feed treated with a commercially available rat bait (brodifacoum, active ingredient). Acceptable recoveries were also obtained from ground beef treated with commercial formulations of diphacinone (67 ± 8%, n = 3) and brodifacoum (83 ± 8%, n = 3). A similar LOQ was observed to that for treatment with analytical chemical. Lower levels could be obtained if necessary, as the bait shavings could be selectively removed from the feed and beef for analysis. In contrast, recoveries from prepared drink mix were much lower for commercial formulations of diphacinone (31 ± 4%, n = 3) and brodifacoum (1 ± 1%, n = 3) as compared to analytical chemicals. It is possible that greater recoveries would have been attained for the commercial formulations if the solutions were heated for a longer time. Extraction of the solid residue remaining after decanting the drink mix resulted in additional recovery, 24% for diphacinone and 18% for brodifacoum.

In conclusion, the seven anticoagulant coumarin and indandione rodenticides could be separated using a C8 column, with a total run time of <29 min. The developed method is relatively rapid. The total time for extraction and sample preparation was <90 min. The two diastereoisomers for bromadiolone could also be separated on the column. In negative ion mode, all chemicals produced a [M − H]⁻ signal and two parent−daughter transitions, which could be used for confirmation and quantitation. Recoveries of animal feed and beef treated with rodenticide were quantitative (recovery > 61%) for all chemicals. The method was also acceptable for five of the seven chemicals in a commercial, powdered, flavored drink mix. The estimated LOQ was <5 ng/g. The developed LC/ESI/MS/MS method was rapid

and provided the simultaneous quantification and confirmation of the seven anticoagulant rodenticides and will aid in the diagnosis and treatment of anticoagulant poisoning.

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