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Quantitation of Eight Anticoagulant Rodenticides in Liver

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DISCLAIMER

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ABSTRACT

Target analytes comprised two chemical classes: hydroxycoumarins (warfarin, coumachlor, dicoumarol, bromadiolone, brodifacoum, and difethialone) and indanediones (diphacinone and chlorophacinone).Liver extracts were cleaned using dispersive solid phase extraction (d-SPE) to remove matrix interferences and analyzed by reverse phase ultraperformance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS). Electrospray ionization in negative ion mode combined with multiple reaction monitoring (MRM) using a triple quadrupole mass spectrometer provided simultaneous confirmation and quantitation. Detection limits spanned 0.75−25 ng/g, and lower quantitation limits were established as 50 ng/g.

Method validation/evaluation/verification:

In-house method validation data and evaluation by an independent laboratory (Vet-LIRN) are published:
https://pubs.acs.org/doi/10.1021/acs.jafc.7b02280

ATTACHMENTS

12-27-23 version Protocols.io Anticoagulant Rodenticides in Liver.pdf

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We use this protocol and it's working

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MATERIALS

A. Equipment / Instrumentation

1. Analytical balance (Sartorius AG BP221S)
2. Geno/Grinder and corresponding ball bearings (SPEX Sample Prep)
3. Vortex (Vortex Genie 2, Fisher Scientific)
4. Reciprocating Shaker (MaxQ 2500, Fisher Scientific)
5. Multi-Mixer & Rotator (MTR 22, United Products & Instruments, Inc.)
6. Centrifuge with rotors suitable for 15-mL and 50-mL centrifuge tubes (Sorvall Legend RT+, Thermo Scientific)
7. Evaporation System with water bath (N-EVAP, Organomation Associates)
8. Tabletop Ultrasonic Cleaner (Model FS-30, Fisher Scientific)
9. Thermo Scientific / Dionex UltiMate 3000 Rapid Separation Liquid Chromatography (UPLC) system with autosampler, binary pump and thermostatted column compartment
10. Thermo Scientific TSQ Quantum Access Max triple quadrupole mass analyzer with electrospray ionization
11. Analytical Chromatography Column (Accucore C18, 2.1 x 100 mm; 2.6 um; Thermo Scientific #17126-102130)
12. Guard Column Cartridge System (UNIGUARD Defender Accucore C18 cartridges; 2.1x10mm, 2.6 um, Thermo Scientific #17126-012105)

B. Supplies

1. Volumetric glassware including flasks (5-mL) and graduated cylinders (10-, 25-, 250-mL)
2. Sharp scissors and forceps
3. Micropipettors and corresponding pipette tips (0.5 uL to 1000 uL)
4. Disposable centrifuge tubes (15-mL; polypropylene; Fisher Scientific #14-959-70C)
5. Disposable centrifuge tubes (50-mL; polypropylene; VWR International #89401-562)
6. Disposable Luer-lok syringes, (5-mL; Fisher Scientific #14-829-45)
7. Disposable microbeakers (5-mL; polystyrene; Fisher Scientific #08-732-119)
8. Syringe filters (PTFE; 0.22 um, 13 mm diameter; Fisher Scientific #50-014-52582)
9. Autosampler vials (2-mL; silanized; amber glass; Fisher Scientific #03-377F)
10. Vial closures and septa (Fisher Scientific #03-379-113)

C. Reagents and Chemicals

1. Organic solvents(HPLC grade; Fisher Scientific):
   a. Methanol (#A452-4),

b. Acetonitrile (#A298-4),
c. Acetone (#A949-4),
d. Chloroform (#C606-4)

2. Ammonium acetate, HPLC grade (Fisher Scientific #A639-500)
3. Ammonium hydroxide, ACS grade (#BDH3014-500MLP, VWR International)
4. Standard Reference Materials:
   a.Coumachlor, 98% (Sigma-Aldrich #189219-1G)
   b.Dicoumarol, 98.0% (Sigma-Aldrich #M1390-5G)
   c.Brodifacoum, 98.1% (US EPA National Pesticide Standard Repository)
   d.Bromadiolone, 98.9% (US EPA National Pesticide Standard Repository)
   e.Chlorophacinone, 99.8% (US EPA National Pesticide Standard Repository)
   f.Difethialone, 99.7% (US EPA National Pesticide Standard Repository)
   g.Diphacinone, 99.3% (US EPA National Pesticide Standard Repository)
   h.Warfarin, 99.5% (US EPA National Pesticide Standard Repository)
5. d-SPE sample clean-up tubes containing 175mg $MgSO_4$,100mg Florisil, 50mg Alumina Basic and 50mg Primary Secondary Amine (UCT #ECQUUS1215CT)

Prepare Solutions

1 [M] 10 % (v/v) Methanol in Acetonitrile: Dilute 25 mL methanol to 250 mL with acetonitrile.

2 Primary Stock Solutions – 1000 ug/mL: For each anticoagulant rodenticide, dissolve 5.0 mg +/-0.1 mg standard reference material in 5 mL of the appropriate solvent (see Table 1), using a 5-mL volumetric flask. These eight solutions should be stored at -20°C and can be stored for up to one year.

Table 1: Solvent Selection Guide

	A	B
	Anticoagulant Rodenticide	**Solvent**
	Bromadiolone, Coumachlor, Warfarin	Methanol
	Brodifacoum, Chlorophacinone, Difethialone, Diphacinone	Acetone
	Dicoumarol	Chloroform

3 Secondary Stock Solution – 10 ug/mL : Transfer 50 µL of each primary stock solution to a single 5-mL volumetric flask. Complete the volume with methanol to prepare a single solution that is 10 ug/mL of each ACR. This solution should be stored at -20°C and can be stored for up to one month.

> **Note**
>
> *The use of a positive displacement pipette may be necessary for accurate transfer of acetone- and chloroform-containing solutions*

4 Mobile Phase Solutions: De-gas mobile phase solutions by helium sparging as needed.

4.1 a. 0.01M Ammonium Acetate, pH 9 – Dissolve 0.77 g +/- 0.01 g ammonium acetate in ~750ml distilled, deionized water (DDI water) in a 1-L volumetric flask. Adjust pH to 9 by adding ammonium hydroxide dropwise. Complete volume with DDI water. This solution may be stored at room temperature for up to one month.

4.2 b. Methanol, HPLC grade - This solution may be stored at room temperature for up to 5 years.

Prepare Matrix-Matched Calibrants and Quality Control (QC) Samples

5 Transfer 1.0 g +/- 0.1 g pre-homogenized control liver to a 50-mL disposable centrifuge tube compatible with the Geno/Grinder. Repeat this step for a total of 7 calibrants and 3 QC samples.

6 Pipette out the appropriate volume of Secondary Stock Solution, as described in Table 2, into the corresponding tube and directly into the homogenized liver.

Table 2: Calibrant and Control Sample Preparation Guide

	A	B	C
	Calibrant / QC Sample	**Concentration (ppb; ng/g)**	**Volume of Secondary Stock Solution (uL)**
	Cal 1	25	2.5
	Cal 2	50	5
	Cal 3	75	7.5
	Cal 4	100	10
	Cal 5	500	50
	Cal 6	1000	100
	Cal 7	2500	250
	QC Blank	0	0
	QC 50 ppb	50	5
	QC 1000 ppb	1000	100

7 Cap tubes and vortex mix the spiked liver for 00:00:10 to mix thoroughly. 10s

8 Proceed to step 10

Sample Homogenization and Extraction

9 Transfer 1.0 g +/- 0.1 g unknown sample liver to a 50-mL disposable centrifuge tube.

10 Homogenize the calibrants, QC samples, and unknown samples as follows:

10.1 Add a 9.5mm steel ball bearing to each tube and cap.

10.2 Grind tissue at a rate of 650 rpm for 00:05:00. One round of impact grinding may be sufficient, however a second round of grinding may be performed if needed. 5m

11 Add 6 mL [M] 10 % (v/v) methanol in acetonitrile to each tube and vortex mix for 00:00:10 to mix thoroughly. 10s

12 Repeat the homogenization process as described in step 10.2.
A single repetition of impact grinding at 650 rpm for 00:05:00 is usually sufficient. 5m

13 Load all calibrants, QC samples, and unknown samples on the reciprocating shaker for 00:30:00. 30m

14 Centrifuge at 829g (2000 RPM using the specified centrifuge) for 00:05:00 5m

Sample Clean-up with d-SPE

15 Transfer the supernatant of each extraction mixture to the corresponding d-SPE tube by pouring carefully and recap the d-SPE tube.

16 Vortex for 00:00:10 to wet the sorbent material completely. 10s

17 Load all calibrants, QC samples, and unknown samples on the multi-mixer & rotator for 00:30:00. 30m

18 Centrifuge at 829g (2000 RPM using the specified centrifuge) for 00:05:00. 5m

Solvent Exchange / Concentrating Extracts

19 Transfer the supernatant of the d-SPE clean-up mixture to a 15-mL disposable centrifuge tube.

Note

The supernatant may be carefully poured from the d-SPE tube to the clean tube.

20 Evaporate to dryness under a gentle stream of nitrogen at 45°C.

Note

Near the end of the drying process, the solution may foam. As long as no bulk solution exists and foam is of negligible volume, the sample preparation process can continue.

21 Transfer 1.0 mL methanol to each tube and vortex for 00:00:10. Sonicate in a water bath for 5m 10s more than 00:05:00 to completely dissolve the residue. Required time for sonication may vary based on unique sample characteristics.

22 Transfer each prepared solution to a clean disposable microbeaker.

23 Draw up the entire volume into a clean 5-mL Luer-lok syringe.

24 Filter the sample through a 0.22 um PTFE syringe filter into an autosampler vial for analysis.

UHPLC – MS/MS Analysis

34m

25 **UHPLC Settings**

Gradient Elution Profile: Profile parameters may be adjusted slightly at the discretion of the analyst to achieve baseline resolution of brodifacoum and difethialone at 2500ppb (Cal 7). The suggested gradient profile is in Table 3.

Table 3: Gradient elution profile

	A	B	C
	Time (min)	**0.01M Ammonium Acetate, pH 9 (%)**	**Methanol (%)**
	0	60	40
	1	60	40
	9	43	57
	15	23	77
	18	19	81
	19	10	90
	24	10	90

	A	B	C
	25	60	40
	34	60	40

25.1 Flow Rate: 0.300 mL/min

25.2 Column Temperature: 25 °C

25.3 Injection Volume: 1 µL

25.4 Total Run Time: 00:34:00 34m

26 MS/MS Detection

These parameters are suggested settings and may need to be optimized for different MS instruments

26.1 ESI Source Conditions:Optimized on the basis of direct infusion of solvent-diluted reference standards

a. Negative ion mode
b. Spray Voltage: 4000 V
c. Vaporizer Temperature: 380 °C
d. Sheath Gas Pressure: 50 psi
e. Auxillary Gas Pressure: 45 psi

f. Ion Sweep Gas Pressure: 0 psi

g. Capillary Temperature: 300 °C

h. Skimmer Offset: (Not used)

26.2 Other Parameters

a. Collision Gas Pressure: 1.7 mTorr

b. Collision Energy: Ion-Dependent; see MRM Transitions Table (Table 4)

c. Tube Lens: Ion-Dependent; see MRM Transitions Table (Table 4)

d. Q1 / Q3 Peak Width (FWHM): 0.70 u

e. Cycle Time: 0.300 s

Table 4. MRM Monitored Transitions / Expected Retention Times

	A	B	C	D	E	F
	Anticoagulant Rodenticide	Retention Time (min)	Precursor Ion ((M-H+)-; u)	Fragment Ion (u)	Collision Energy (eV)	Tube Lens (V) Tube Lens (V)
	Warfarin	2.00	307	161*	22	70
			307	250	25	70
	Coumachlor	4.87	341	284	26	71
			341	161	23	71
	Diphacinone	7.48	339	167	28	77
			339	165	48	77
	Dicoumarol	7.68	335	161	21	47
			335	117	47	47
	Chlorophacinone	11.08	373	201	24	76
			373	145	25	76
	Bromadiolone**	13.76	525	250	38	97
			525	273	40	97
	Brodifacoum	16.28	521	135	40	101
			521	143	57	101
	Difethialone	16.53	537	151	41	100
			537	371	35	100

*Transitions in bold are used for quantitation
**Two isomers are present for Bromadiolone; only the earliest eluting (and most abundant) isomer is used for detection and quantitation

27 Post-Acquisition Data Analysis

Note

Automated peak integration should be used as much as possible to minimize manual data integration. Automated integration parameters should be determined based upon those that provide accurate peak integration in sample and calibrant chromatograms and may vary between analytes.

27.1 Qualitative Identification: Comparisons of unknown chromatograms to those of calibrants and QC samples are used to determine the presence or absence of each analyte. For an analyte to be considered present in a sample, the following requirements must be met:

a. Retention Time:

1. The retention times of the primary (Quan) and secondary (Confirming) ions are within 0.25 min of the mean retention time for the corresponding analyte in all calibrants and overspiked QC samples acquired within the same batch.
2. The primary (Quan) ion and the corresponding secondary (Confirming) ion co-elute within 0.1 min of one another.

b. Signal Intensity:

1. The peak areas in the primary and secondary ion channels must have a S/N ratio of 3 or greater.
2. If there are detectable AR levels in any of the associated blank samples (e.g. instrument blanks, reagent blanks, and negative control samples), then the peak area in the unknown sample must be at least 10 times that measured in the blank sample.

c. Ion Ratio Measurement: The ion ratios of the primary to secondary ions for a given analyte, expressed as a percentage, must be within 20% of the average ion ratio for the associated spiked matrix-matched samples (e.g. calibrants, QC samples) acquired concurrently.

27.2 Quantitative Analysis: Recommended method

Perform linear least squares regression using peak areas for all calibrants versus concentration, ranging from 25 to 2500 ppb. The following parameters should be used to generate calibration curves to determine quantitative results:

a. Weighting $1/x^2$

b. Ignore Origin

c. The coefficients of determination (R^2) for each calibration are expected to be ≥0.985 when the fit is linear and the origin (0,0) is ignored.

Note

Occasionally, a quadratic instrument response is observed at concentrations > 1000 ppb. It is permissible to use a quadratic trendline, with or without weighting, in these situations with an R^2 value of 0.985 or better.

d. The intensity of the quantifying ion is greater than the intensity of the same ion in the least concentrated calibrant.

Note

Overspiked QC samples (e.g., 50 ppb and 1,000 ppb) must yield AR concentration results that are within acceptable range (e.g., +/- 15%) of the expected AR concentrations