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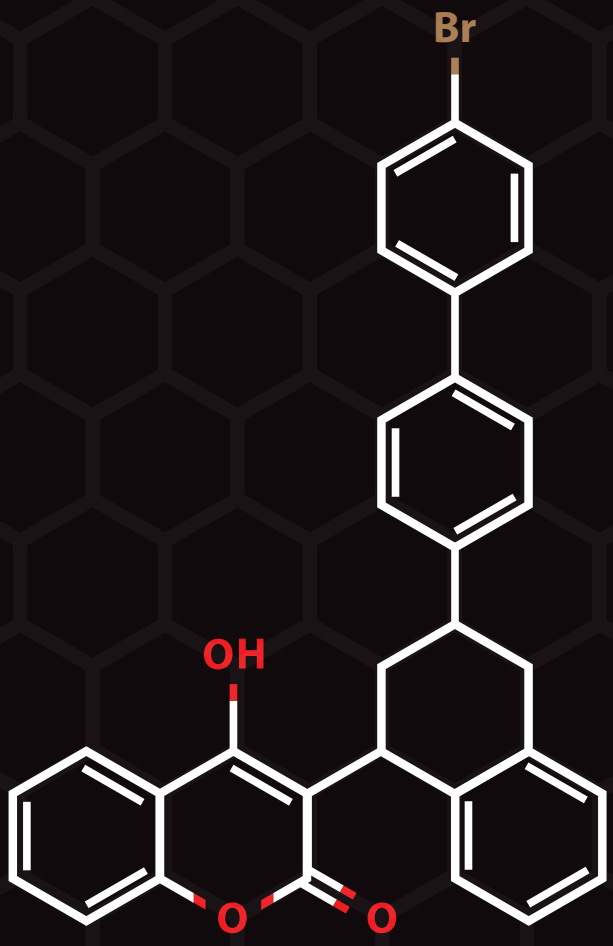
Superwarfarin Toolkit

June 2022



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NIJ | National Institute of Justice

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ACKNOWLEDGEMENTS:

This report was prepared by Hiu Yu Lam, MS; Tais R. Fiorentin, PhD; Francis X. Diamond, BS; Amanda L.A. Mohr, MS; Barry K. Logan, PhD.

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PURPOSE: The Superwarfarin Toolkit is a consolidation of our grant research outcomes into a comprehensive document detailing relevant information about the characterization of anticoagulant compounds.

This toolkit includes basic drug information, chemical structure, clinical effects, forensic casework regarding anticoagulant, methods for identification and confirmation, and much more. This toolkit is designed to serve as a one-stop resource for scientists and interested individuals looking for all-inclusive information about anticoagulant compounds.

ABOUT US: The Center for Forensic Science Research and Education (CFSRE, Willow Grove, PA) is a non-profit organization that operates a state-of-the-art laboratory with a mission to advance forensic science testing and knowledge. In 2019, the National Institute of Justice had awarded and funded this project entitled "Identification of Anticoagulant Adulterants in Seized Material and Biological Samples."

To date, the assessment of current methods in seized materials have been performed, as well as development of new, unique screening and confirmation methodologies for anticoagulant compounds.

*CFSRE welcomes collaborative partnerships with engaged agencies and communities impacted by anticoagulant related cases. Individuals can contact our program to learn more about anticoagulants, our advanced testing capabilities, to request information regarding sample submissions, and/or to join our growing dissemination networks.

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EMERGENCE OF ANTICOAGULANT ADULTERANTS IN SYNTHETIC CANNABINOIDS

PURPOSE: The objective of this announcement is to notify public health and safety, law enforcement, first responders, clinicians, medical examiners and coroners, forensic and clinical laboratory personnel, and all other related communities about the adulteration of synthetic cannabinoid material with anticoagulants.

BACKGROUND: Warfarin and superwarfarins are anticoagulant agents widely used in commercial rodenticides and in the treatment of bleeding disorders. The major compounds of the class include brodifacoum, bromadiolone and difenacoum, all of which have a history of involvement in many types of forensic casework such as suicides, homicides, and accidental and deliberate poisonings. They have also been identified as potential chemical terror agents. Currently, these anticoagulant compounds are not regulated nor controlled within the United States. Individuals can purchase these compounds within United States or purchase overseas and import into United States without restriction. Toxic clinical effects of anticoagulant exposure include spontaneous internal and external bleeding, and can lead to death. Due to these anticoagulant drugs' dramatic effects, toxicity, long half-life, as well as the difficulty of diagnosing and treating patients, the adulteration of seized drug material with anticoagulants is a serious public health concern.

Recently, these substances have emerged as toxic adulterants in synthetic cannabinoid, cocaine and marijuana casework, and have led to hundreds of hospitalizations and several deaths in the United States. In 2018 in Chicago, Illinois, there was an outbreak involving a large number of synthetic cannabinoids laced with brodifacoum. More than 150 individuals that consumed these laced synthetic cannabinoid products were hospitalized with the presence of coagulopathy and bleeding symptoms, and five individuals died from major bleeding events. A similar anticoagulant outbreak occurred at Tampa, Florida in December 2021, which resulted in more than 50 individuals hospitalized and two deaths.

GOALS AND OBJECTIVE: With the support of the National Institute of Justice, the CFSRE performed a study gap analysis involving a systematic evaluation of current routine approaches to the examination and characterization of anticoagulant-containing materials, including commercial baits used in rodent control, anticoagulant laced/adulterated street drugs, and the development of workflows for screening and confirmation/quantitation of the drugs in toxicological samples for ten common anticoagulant chemicals available in the United States. Provided in this toolkit are practical analytical approaches and resources for the analysis of anticoagulant adulterants in forensic casework for forensic laboratories with a range of resources and technology.

RECOMMENDATIONS FOR PUBLIC HEALTH:

- Implement surveillance for rapid identification of anticoagulant drug overdose outbreaks.
- Engage local poison centers and clinicians to assist with treatment of affected patients.
- Track and monitor geographical drug laced with anticoagulant compounds distribution and trends.
- Track demographics and known risk factors for decedents and overdose patients.
- Raise awareness about the risks and dangers associated with toxic anticoagulant adulteration.

RECOMMENDATIONS FOR LABORATORIES:

- Utilize analytical data available publicly for the identification of anticoagulant compounds if reference standards are not available.
- Develop a sensitive and selective testing procedure/methodology for anticoagulant drugs.
- Prioritize analytical testing of seized materials or toxicology samples obtained from overdoses that are suspected to be anticoagulant related for investigations.
- Share data on outbreaks associated with drug products being laced with anticoagulant drugs with local health department, medical examiners, and related communities.

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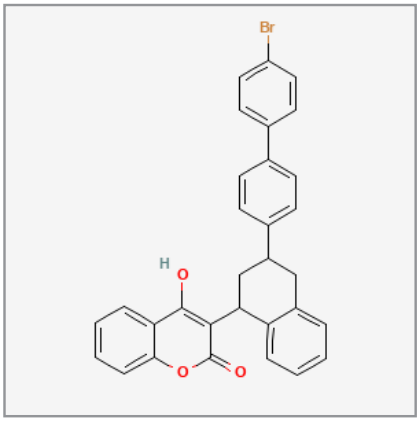
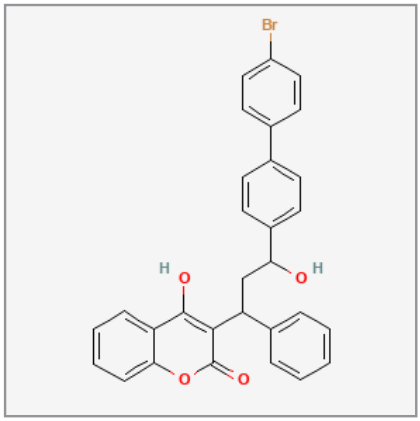
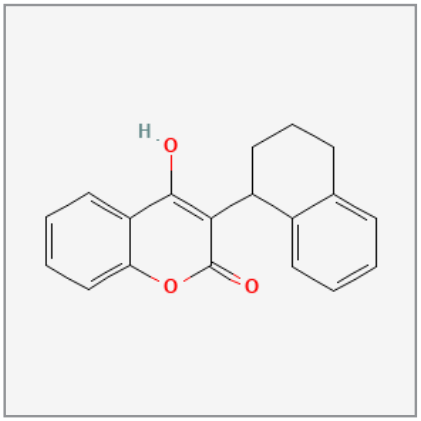
Brodifacoum $C_{31}H_{23}BrO_3$



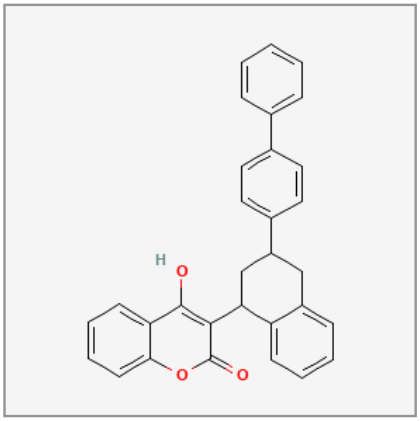
Bromadiolone $C_{30}H_{23}BrO_a$



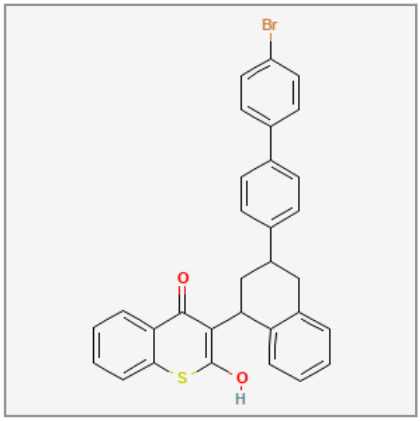
Coumatetralyl $C_{19}H_{16}O_3$



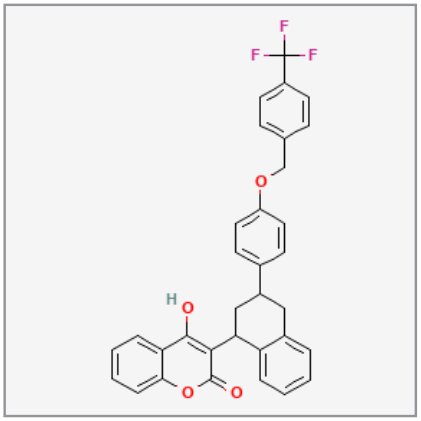
Difenacoum $C_{31}H_{24}O_3$



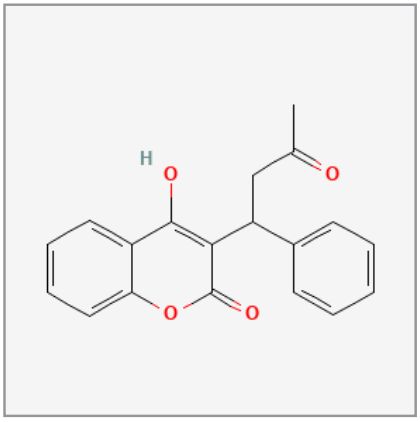
Difethialone $C_{31}H_{23}BrO_2S$



Flocoumafen $C_{33}H_{25}F_3O_4$



Warfarin $C_{19}H_{16}O_4$



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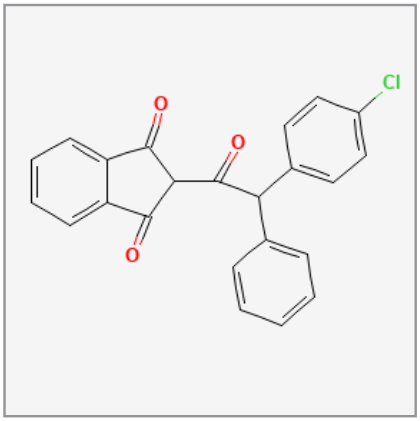
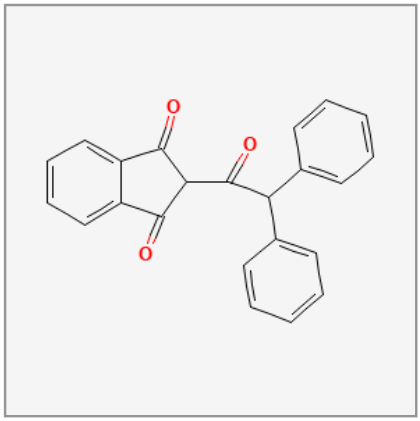
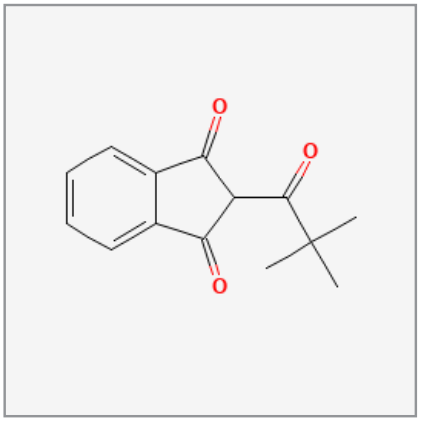
Chlorophacinone $C_{23}H_{15}ClO_3$



Diphacinone $C_{23}H_{16}O_3$



Pindone $C_{14}H_{14}O_3$



Presumptive tests in forensic chemistry analysis are usually performed by color tests. Ten anticoagulant drugs were evaluated using the most common test reagents: Cobalt Thiocyanate, Dille-Koppanyi, Duquenois-Levine, Mandelin's, Marquis, Froehde and Mecke. The color changes and photographs are shown on pages 5-9.

		WARFARIN 1 mg/mL	DIPHACINONE 1 mg/mL	COUMATETRALYL 1 mg/mL	BROMADIOLONE 1 mg/mL	DIFENACOUM 1 mg/mL
Cobalt Thiocyanate	Color	No color	No color	No color	No color	No color
	Change Observed	**BLUE (→ PINK)**	**BLUE (→ PINK)**	**BLUE (→ PINK)**	**NO**	**BLUE (→ PURPLE)**
Dille-Koppanyi	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**NO**	**NO**
Duquenois-Levine	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**NO**	**NO**
Mecke	Color	No color	No color	No color	No color	No color
	Change Observed	**PINK**	**BROWN**	**BROWN**	**LT PINK (→ BROWN)**	**NO**
Marquis	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**YELLOW**	**ORANGE**	**ORANGE**	**NO**
Froehde	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**YELLOW**	**NO**	**PINK**	**LIGHT YELLOW**
Mandelin's	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**BROWN**	**BLACK**

		BRODIFACOUM 1 mg/mL	DIFETHIALONE 0.01 mg/mL	FLOCOUMAFEN 1 mg/mL	PINDONE 1 mg/mL	CHLOROPHACINONE 1 mg/mL
Cobalt Thiocyanate	Color	No color	No color	No color	No color	No color
	Change Observed	**BLUE (→ PURPLE)**	**BLUE (→ PINK)**	**BLUE**	**BLUE (→ GREY)**	**NO**
Dille-Koppanyi	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**NO**	**NO**
Duquenois-Levine	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**NO**	**NO**
Mecke	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**DARK YELLOW**	**NO**	**YELLOW**	**YELLOW**
Marquis	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**YELLOW**	**YELLOW**
Froehde	Color	No color	No color	No color	No color	No color
	Change Observed	**NO**	**NO**	**NO**	**YELLOW**	**YELLOW**
Mandelin's	Color	No color	No color	No color	No color	No color
	Change Observed	**BLACK**	**YELLOW**	**NO**	**GREEN**	**YELLOW**

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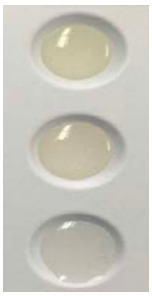
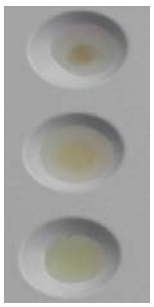
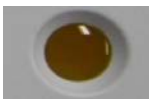
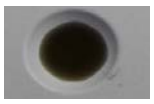
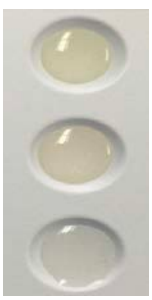
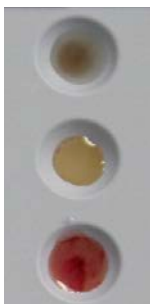
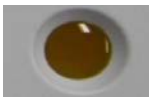
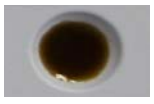
ANALYTICAL STANDARD	COLOR TEST		REACTION
BRODIFACOUM 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

ANALYTICAL STANDARD	COLOR TEST		REACTION
BROMADIOLONE 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

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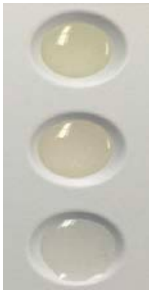
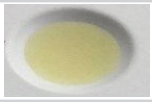
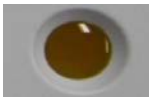
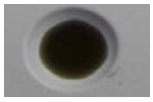
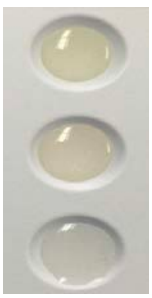
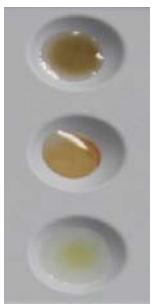
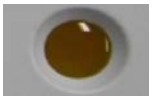
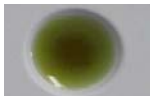
ANALYTICAL STANDARD	COLOR TEST		REACTION
CHLOROPHACINONE 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

ANALYTICAL STANDARD	COLOR TEST		REACTION
COUMATETRALYL 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

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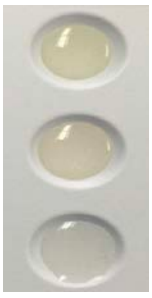
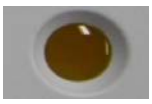
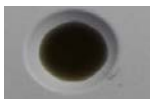
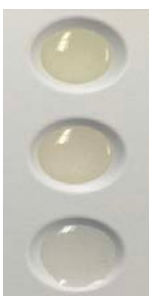
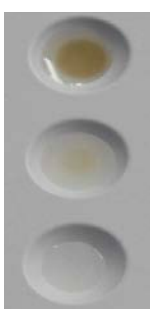
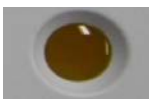
ANALYTICAL STANDARD	COLOR TEST		REACTION
DIFENACOUM 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

ANALYTICAL STANDARD	COLOR TEST		REACTION
DIFETHIALONE 0.01 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

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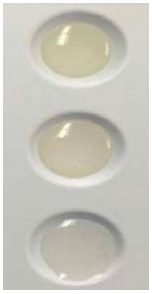
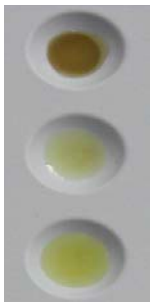
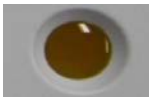
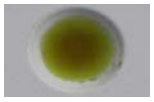
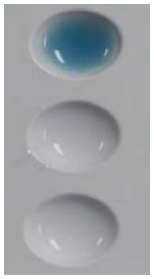
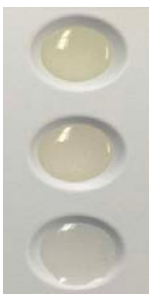
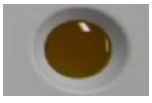
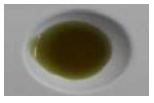
ANALYTICAL STANDARD	COLOR TEST		REACTION
DIPHACINONE 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

ANALYTICAL STANDARD	COLOR TEST		REACTION
FLOCOUMAFEN 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

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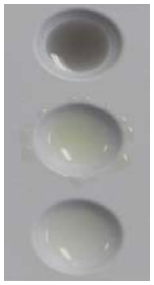
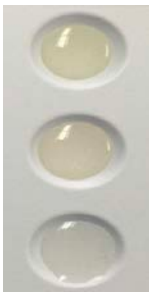
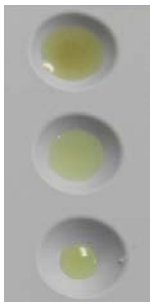
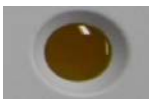
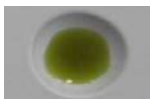
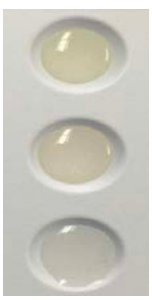
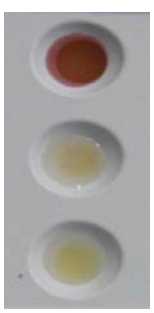
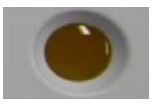
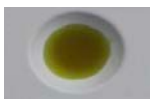
ANALYTICAL STANDARD	COLOR TEST		REACTION
PINDONE 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

ANALYTICAL STANDARD	COLOR TEST		REACTION
WARFARIN 1 mg/mL	Cobalt Thiocyanate		
	Dille-Koppanyi		
	Duquenois-Levine		
	Mecke		
	Marquis		
	Froehde		
	Mandelin's		

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Ten commercial products were selected to evaluate and characterize. The commercial products evaluated were selected at random based on their availability and active ingredient. The table below shows the commercial products and respective active ingredients. The color changes and photographs for commercial products are shown on pages 12-16.

NAME	BRAND	ACTIVE INGREDIENT (PERCENT COMPOSITION)
Ramik Green Nuggets	Neogen	Diphacinone (0.005%)
DryUp Bars	Harris	Diphacinone (0.005%)
Just One Bite II Bar	Farnam	Bromadiolone (0.005%)
Havoc-XT Blok	Neogen	Brodifacoum (0.005%)
Ditrac All-Weather Blox	Bell	Diphacinone (unknown)
TomCat All-Weather Bait Chunk	Motomco	Diphacinone (unknown)
Bait Block Peanut Butter	JT Eaton	Diphacinone (unknown)
d-Con Bait Blocks	d-Con	Diphacinone (0.005%)
Rodentex Multi-feed Bars	Farnam	Diphacinone (0.005%)
Rodex Pelleted Bait-1	Neogen	Warfarin (0.025%)

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		Ramik Green Nuggets AI: diphacinone	**DryUp Bars** AI: diphacinone	**Just One Bite II Bar** AI: bromadiolone	**Havoc-XT Blok** AI: bromadiolone	**Ditrac All-Weather Blox** AI: diphacinone
Cobalt Thiocyanate	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**Blue**	**No**	**No**	**No**	**No**
Dille-Koppanyi	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**No**	**No**	**No**	**No**	**No**
Duquenois-Levine	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**No**	**No**	**No**	**No**	**No**
Mecke	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**Brown**	**Brown**	**Brown**	**Yellow**	**Brown**
Marquis	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**Brown**	**Yellow**	**No**	**Yellow**	**Yellow**
Froehde	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**Brown**	**Brown**	**No**	**Yellow**	**Brown**
Mandelin's	Color	Green	Light Yellow	Yellow	Blue	Light Green
	Change Obs.	**Brown**	**No**	**No**	**No**	**Dark Green**

		TomCat All-Weather Bait Chunk AI: diphacinone	**Bait Block Peanut Butter** AI: diphacinone	**d-Con Bait Blocks** AI: diphacinone	**Rodentex Multi-Feed Bars** AI: diphacinone	**Rodex Pelleted Bait-1** AI: warfarin
Cobalt Thiocyanate	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**No**	**No**	**No**	**No**	**No**
Dille-Koppanyi	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**No**	**No**	**No**	**No**	**No**
Duquenois-Levine	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**No**	**No**	**No**	**No**	**No**
Mecke	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**Brown**	**Brown**	**No**	**Brown**	**No**
Marquis	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**Light Brown**	**Brown**	**No**	**No**	**No**
Froehde	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**Light Brown**	**Brown**	**No**	**Brown**	**No**
Mandelin's	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change Obs.	**Dark Green**	**Dark Blue**	**No**	**No**	**No**

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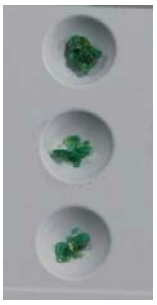
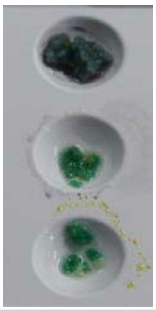
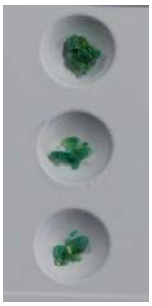
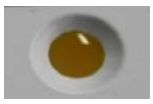
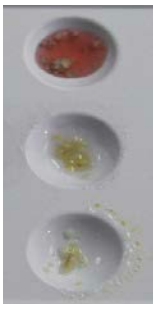
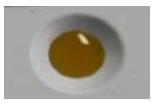
COMMERCIAL PRODUCT		COLOR TEST		REACTION
RAMIK GREEN NUGGETS AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

COMMERCIAL PRODUCT		COLOR TEST		REACTION
DRYUP BARS AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

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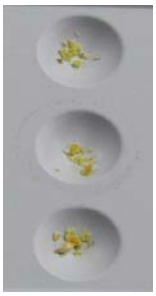
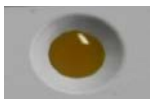
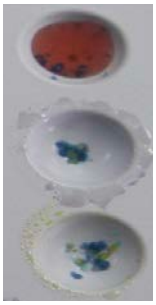
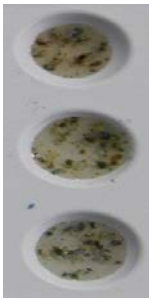
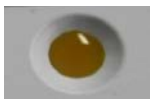
COMMERCIAL PRODUCT		COLOR TEST		REACTION
JUST ONE BITE II BAR AI: bromadiolone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

COMMERCIAL PRODUCT		COLOR TEST		REACTION
HAVOC-XT BLOK AI: bromadiolone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

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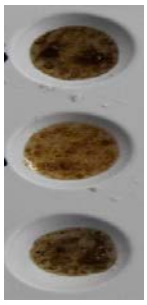
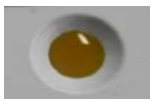
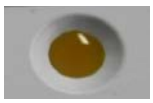
COMMERCIAL PRODUCT		COLOR TEST		REACTION
DITRAC ALL-WEATHER BLOX AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

COMMERCIAL PRODUCT		COLOR TEST		REACTION
TOMCAT ALL-WEATHER BAIT CHUNK AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

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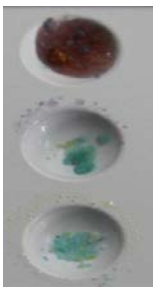
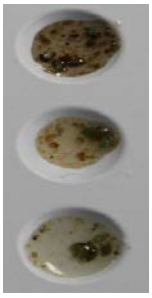
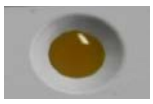
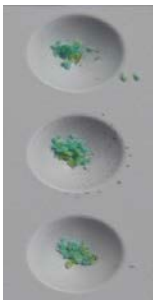
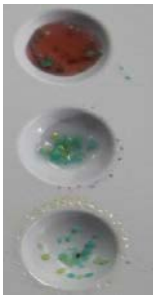
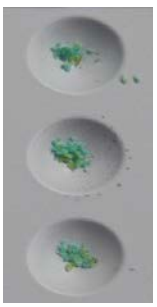
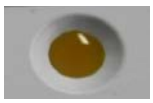
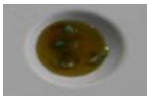
COMMERCIAL PRODUCT		COLOR TEST		REACTION
BAIT BLOCK PEANUT BUTTER AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

COMMERCIAL PRODUCT		COLOR TEST		REACTION
D-CON BAIT BLOCKS AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

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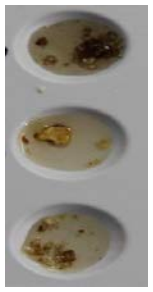
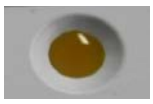
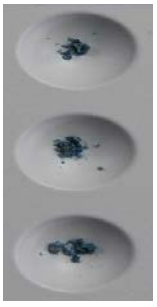
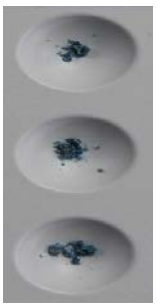
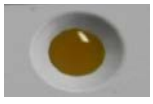
COMMERCIAL PRODUCT		COLOR TEST		REACTION
RODENTEX MULTI-FEED BARS AI: diphacinone		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

COMMERCIAL PRODUCT		COLOR TEST		REACTION
RODEX PELLETED BAIT-1 AI: warfarin		Cobalt Thiocyanate		
		Dille-Koppanyi		
		Duquenois-Levine		
		Mecke		
		Marquis		
		Froehde		
		Mandelin's		

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PURPOSE: To provide an example analytical method for the analysis of dyes in commercial rodenticide products. Many anticoagulant preparations have marker dyes or dye mixtures intermixed to facilitate identification. The HPLC-DAD-UV method below was used to separate and further characterize the dyes in these commercial products.

AGILENT TECHNOLOGIES
HPLC-DAD-UV
HP 1100 SERIES

GRADIENT SETTING	
TIME	%B
0.00	5
1.50	5
3.00	20
6.00	40
10.00	60
13.00	80
16.00	90
18.00	90
21.00	80
24.00	60
27.00	50
30.00	25
32.00	20
35.00	5
40.00	5

LIQUID CHROMATOGRAPH PARAMETERS	
Column	NOVA-PAK 3.9x150mm, 4 µm
Column Temperature	40 ˚C
Mobile Phase A	20mM Ammonium Acetate buffer with 1% Acetic Acid
Mobile Phase B	Acetonitrile
Flow Rate	1 mL/min.
Injection Volume	10 µL

DAD-UV PARAMETERS	
DAD Signals	220, 280, 350, 450, and 650
Spectrum Range	250-800 nm

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PURPOSE: To provide an example analytical method for the analysis of anticoagulant drugs in seized material via gas chromatography mass spectrometry (GC/MS).

AGILENT TECHNOLOGIES (SANTA CLARA, CA)
Gas Chromatograph: 6890 Network GC System
Mass Spectrometer: 5975B inert XL MSD

GAS CHROMATOGRAPH PARAMETERS	
Column	DB-1 (0.20 mm x 12 m; 0.33µm film thickness
He flow rate	2.3 mL/min
Injection Port Temp	265˚C
Injection Volume	1 µL
Mode	Splitless
Inlet Pressure	18.6 psi
Oven	Initial Temp: 50˚C, ramp temp at the rate of 30˚C/min up to 340˚C.
Aux Temp	300˚C

MASS SPECTROMETER PARAMETERS	
Source Temp.	230˚C
MS range	50~750 m/z

DRUG	LOD (mg/mL)
Brodifacoum[1,2]	0.1
Bromadiolone[1]	0.2
Chlorophacinone	0.1
Coumatetralyl	0.01
Difenacoum[1]	0.2
Difethialone[1,2]	0.05
Diphacinone	0.04
Flocoumafen[1]	0.1
Pindone	0.0025
Warfarin	0.02

[1] Identified by the degradation product.

[1,2] Because both analytes yield the same degradation product, they were run individually for diagnostic purposes.

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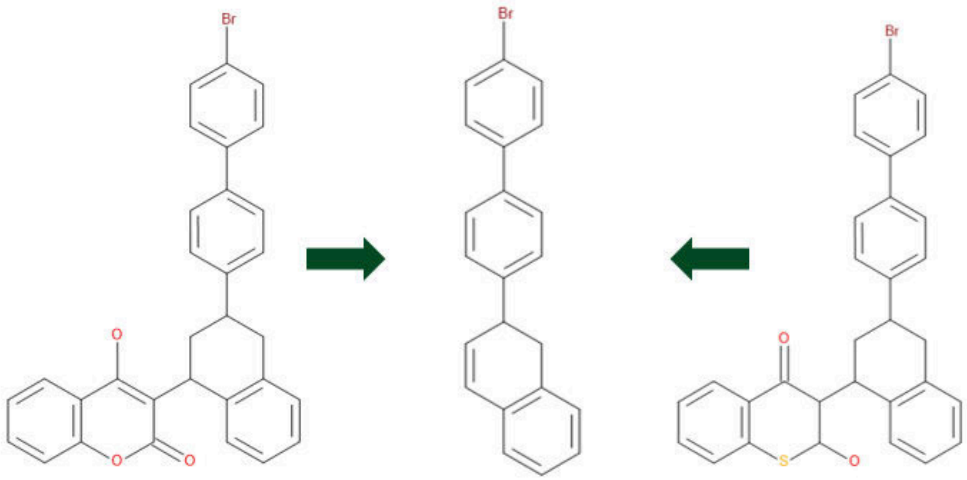
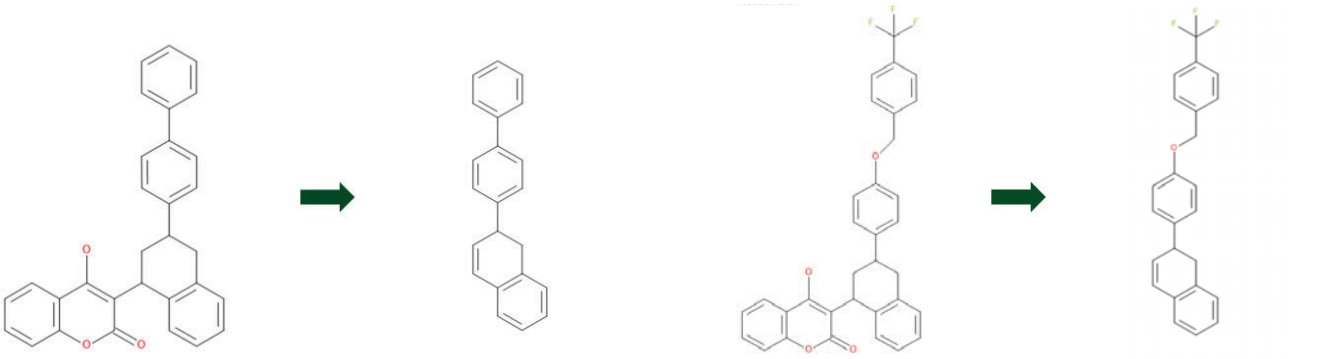
Both brodifacoum and difethialone breakdown into the same degradation product by breaking the naphthalene substrate. Because both drugs have the same degradation product, another analytical technique may be necessary to positively identify brodifacoum and difethialone.



Brodifacoum Degradation Product Difethialone

Difenacoum and flocoumafen follow a similar pattern like brodifacoum and difethialone, where the breaking of the naphthalene substrate forms the degradation product. However, each forms a unique degradation product indicative of the original drug.



Difenacoum Degradation Product Flocoumafen Degradation Product

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PURPOSE: To provide an example analytical method for the analysis of anticoagulant drugs in seized material via liquid chromatography ion trap mass spectrometry (LCTRAP).

THERMO FISHER (WALTHAM, MA)
Liquid Chromatograph: Vanquish UHPLC System
Mass Spectrometer: QExactive Hybrid Quadrupole-Orbitrap Mass Spectrometer

MASS SPECTROMETER PARAMETERS	
Spray Voltage	2.5 kV
Capillary Temp.	300 °C
AGC Target	1e5
Resolution	35,000
Stepped Collision Energy	20, 40, and 80 eV

LIQUID CHROMATOGRAPH PARAMETERS	
Column	Accucore C18 (2.1 x 100 mm, 2.6µm)
Column Temp.	30 °C
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.3 mL/min
Gradient	0 min: 95:5 A:B
	3 min: 40:60 A:B
	7 min: 5:95 A:B
	7.01 min: 95:5 A:B
	8.50 min: 95:5 A:B

COMPOUND (POLARITY)	PRECURSOR (m/z)	FRAGMENT IONS (m/z)
Brodifacoum D4 (+)	527.1154	335.0427, 256.1243, 178.0776, 165.0699, 91.0548
Brodifacoum (+)	523.0903	335.0420, 256.1245, 178.0777, 165.0700, 91.0550
Bromadiolone (-)	525.0707	283.0420, 250.0600, 163.0200, 93.0300, 78.9200
Chlorophacinone (+)	375.0783	321.0907, 263.0700, 235.075, 178.0775, 165.0700
Coumatetralyl (+)	293.1172	175.0390, 121.0286, 107.0494, 91.0550, 79.0548
Difenacoum (+)	445.1798	257.1320, 179.8500, 178.0777, 165.0700, 91.0548
Difethialone (+)	539.0675	335.0426, 256.1242, 178.0776, 165.0698, 91.0547
Diphacinone (+)	341.1172	323.1063, 263.0700, 235.0750, 178.0776, 105.0340
Flocoumafen (+)	543.1778	523.1711, 355.1302, 291.1010, 159.0420, 109.0451
Pindone (+)	231.1016	213.0909, 185.0960, 165.0699, 152.0619, 128.0620
Warfarin (+)	309.1121	251.0700, 191.0337, 163.0390, 147.0800, 121.0286

METHOD CHARACTERISTICS	
Limit of Detection	100 ng/mL
Carryover	>10,000 ng/mL for chlorophacinone, difenacoum, and diphacinone; >100,000 ng/mL for the remaining compounds
Autosampler Stability (at -15 °C)	All compounds were stable for up to 72 hours with the exception of brodifacoum, chlorophacinone and bromadiolone. Chlorophacinone and brodifacoum at the low concentration (100 ng/mL) were unstable after 24h and bromadiolone after 48h.

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PURPOSE: To provide two example sample preparation workflows for the isolation of anticoagulant analytes from seized material. These preparation approaches provide a starting point for laboratories looking to assess sample preparation methods for these drugs, ultimately saving valuable time and resources. These sample preparation methods could serve useful for a qualitative screening approach.

ACID BASE EXTRACTION FOR GCMS

1. Add ~50 mg seized material (plant material) to a 13x100 test tube.
2. Add 1 mL of DI water.
3. Add 200 µL of internal standard (brodifacoum D4).
4. Add 3-5 drops of concentrated hydrochloric acid; use pH paper to test acidity.
5. Add 1 mL of 90:10 DCM:IPA extraction solvent.
6. Vortex and transfer the organic layer (bottom layer) to a new 13x100 test tube.
7. To the original 13x100 test tube, add 3-5 drops of concentrated ammonium hydroxide; use pH paper to test basicity.
8. Add 1 mL of 90:10 DCM:IPA extraction solvent.
9. Vortex and transfer the organic layer (bottom layer) to the same test tube in step 6.
10. For GCMS analysis, transfer the liquid to an autosampler vial, cap and crimp.

. . .

METHANOL DILUTION FOR GCMS

1. Add ~50 mg seized material (plant material) to a 13x100 test tube.
2. Add 1 mL of methanol.
3. Add 200 µL of internal standard n-propylamphetamine and 10,11-dihydrodibenz[b,f][1,4]-oxazepin-11-one.
4. Vortex and transfer to an autosampler vial, cap and crimp.

. . .

METHANOL DILUTION FOR LCTRAP

1. Add ~50 mg seized material seized material (plant material) to a 13X100 test tube.
2. Add 1 mL of methanol.
3. Vortex and dilute 1:99 with 95:5 A:B mobile phase (10 µL of methanol solution + 990 µL of mobile phase).
4. Add 50 µL of brodifacoum D4 internal standard.
5. Vortex and transfer 1 mL to an autosampler vial and cap.

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PURPOSE: To provide an example analytical method for the analysis of anticoagulant drugs in biological matrices via liquid chromatography ion trap mass spectrometry (LCTRAP).

THERMO FISHER (WALTHAM, MA)
Liquid Chromatograph: Vanquish UHPLC System
Mass Spectrometer: QExactive Hybrid Quadrupole-Orbitrap Mass Spectrometer

MASS SPECTROMETER PARAMETERS	
Spray Voltage	2.5 kV
Capillary Temp.	300˚C
AGC Target	1e5
Resolution	35,000
Stepped Collision Energy	20, 40, and 80 eV

LIQUID CHROMATOGRAPH PARAMETERS	
Column	Accucore C18 (2.1 x 100 mm, 2.6µm)
Column Temp.	30˚C
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.3 mL/min
Gradient	0 min: 95:5 A:B
	3 min: 40:60 A:B
	7 min: 5:95 A:B
	7.01 min: 95:5 A:B
	8.50 min: 95:5 A:B

COMPOUND (POLARITY)	PRECURSOR (m/z)	FRAGMENT IONS (m/z)
Brodifacoum D4 (+)	527.1154	335.0427, 256.1243, 178.0776, 165.0699, 91.0548
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Warfarin (+)	309.1121	251.0700, 191.0337, 163.0390, 147.0800, 121.0286

METHOD CHARACTERISTICS	
Limit of Detection	100 ng/mL
Carryover	>2,000 ng/mL
Autosampler Stability (at -10˚C)	72 hours for bromadiolone, chlorophacinone, coumatetralyl, difenacoum, diphacinone, flocoumafen and warfarin. 48 hours for brodifacoum. 24 hours for difethialone and pindone.

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PURPOSE: To provide an example sample preparation workflow for the extraction of anticoagulant analytes from human blood.

EXTRACTION PROTOCOL:

PROTEIN CRASH:

1. Aliquot 0.5 mL of blood into a 13x100 test tube
2. Add 25 µL of internal standard (brodifacoum D4)
3. Vortex for 30 seconds
4. Add 0.5 mL of formate buffer (pH 3)
5. Add 2 mL of acetone
6. Cap and rotate for 5 minutes
7. Centrifuge for 5 minutes at 3000 rpm
8. Transfer top layer to a new 13x100 test tube

LIQUID-LIQUID EXTRACTION:

9. Add 1.5 mL of n-butyl chloride
10. Cap and rotate for 5 minutes
11. Centrifuge for 5 minutes at 3000 rpm
12. Transfer top layer to a new 13x100 test tube
13. Evaporate to dryness at 35°C for 25 minutes
14. Reconstitute in 100 µL of mobile phase (95A:5B)

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PURPOSE: To provide an example of an analytical method for the analysis of anticoagulant drugs in biological matrices via liquid chromatography tandem mass spectrometry. This analytical methodology can be used qualitatively and quantitatively.

THERMO FISHER (WALTHAM, MA)
Liquid Chromatograph: Acquity UPLC
Mass Spectrometer: Xevo TQ-S micro QQQ Mass Spectrometer

MASS SPECTROMETER PARAMETERS	
Capillary	3.2 kV
Desolvation Temp.	500˚C
Desolvation Flow Rate	1,000 L/Hr
Ionization mode	Negative

METHOD CHARACTERISTICS	
Linear Range	5-250 ng/mL
Limit of Detection	1 ng/mL
Carryover	>250 ng/mL
Autosampler Stability	72 hours
Applicable Matrices	Blood, Serum, Plasma, Urine

LIQUID CHROMATOGRAPH PARAMETERS	
Column	ACQUITY UPLC BEH C18 (2.1 x 100 mm, 1.7 µm)
Column Temp.	60˚C
Autosampler Temp.	15˚C
Mobile Phase A	0.02% Ammonium Hydroxide in Water
Mobile Phase B	0.02% Ammonium Hydroxide in Methanol
Flow Rate	0.4 mL/min
Gradient	0 min: 95:5 A:B
	1.6 min: 5:95 A:B
	3.5 min: 5:95 A:B
	3.6 min: 95:5 A:B
	4 min: 95:5 A:B

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COMPOUND	TOP: PRECURSOR ION TO QUANTIFICATION ION (m/z) BOTTOM: PRECURSOR ION TO QUALIFIER ION (m/z)	CONE VOLTAGE (V)	COLLISION ENERGY	EXPECTED RT (min)
Brodifacoum D4	527.1 → 139.3 527.1 → 223.2	4 4	40 40	2.01
Brodifacoum	521.0 → 135.0 521.0 → 93.1	6 6	36 62	2.01
Bromadiolone D5	532.2 → 255.2 532.2 → 137.3	4 4	35 35	1.92
Bromadiolone	527.0 → 250.0 525.0 → 250.0	66 18	34 34	1.90
Chlorophacinone D4	377.1 → 201.1 377.1 → 149.0	24 24	20 20	1.80
Chlorophacinone	373.1 → 201.1 373.1 → 145.2	4 4	20 20	1.80
Coumatetralyl D4	295.0 → 141.1 295.0 → 110.0	2 2	24 24	1.49
Coumatetralyl	291.0 → 141.1 291.0 → 106.1	4 4	24 24	1.50
Difenacoum D4	448.2 → 294.2 448.2 → 139.4	4 4	35 35	1.93
Difenacoum	443.3 → 135.2 443.3 → 293.2	4 4	35 35	1.93
Difethialone	537.0 → 79.0 537.0 → 151.0	90 90	42 38	2.02
Diphacinone D4	343.1 → 167.1 343.1 → 176.1	4 4	20 20	1.68
Diphacinone	339.1 → 167.2 339.1 → 172.1	4 4	25 30	1.68
Flocoumafen D4	545.2 → 386.0 545.2 → 165.0	60 60	24 36	1.98
Flocoumafen	541.1 → 381.9 541.1 → 161.0	86 86	22 38	1.98
Pindone	229.4 → 116.1 229.4 → 144.0	4 4	35 25	1.46
Warfarin D5	312.0 → 161.0 312.0 → 255.0	14 14	18 20	1.42
Warfarin	307.0 → 160.0 307.0 → 250.0	56 56	16 20	1.42

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PURPOSE: To provide a unique example of sample preparation workflows for the extraction of anticoagulant drugs from human blood. This extraction protocol can be used for screening or confirmatory testing.

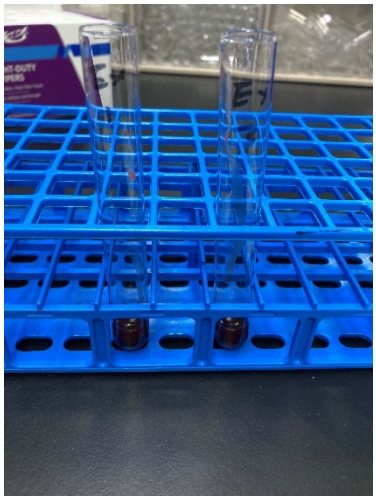
EXTRACTION PROTOCOL:

PROTEIN CRASH:

1. Aliquot 0.5 mL of blood into a 13x100 test tube
2. Add 25 µL of internal standard mix (brodifacoum D4, bromadiolone D5, chlorophacinone D4, coumatetralyl D4, difenacoum D4, diphacinone D4, flocoumafen D4 and warfarin D5).
3. Add 0.5 mL of formate buffer (pH 3)
4. Add 2 mL of acetone
5. Cap and rotate for 5 minutes
6. Centrifuge for 5 minutes at 3000 rpm
7. Transfer top layer into a new 13x100 test tube

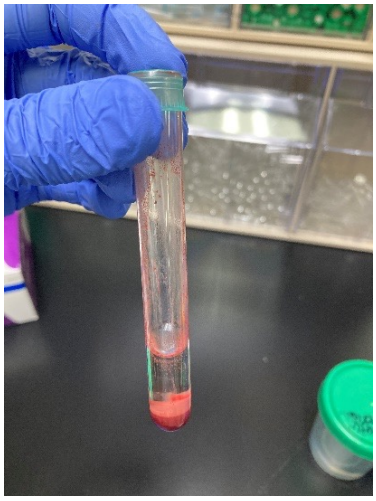
LIQUID-LIQUID EXTRACTION:

8. Add 1.5 mL of n-butyl chloride
9. Cap and rotate for 5 minutes
10. Centrifuge for 5 minutes at 3000 rpm
11. Transfer top layer into a new 13x100 test tube
12. Evaporate to dryness at 35˚C for 25 minutes
13. Reconstitute in 200 µL of 95:5 MPA:MPB
14. Vortex and transfer solution to a Costar® Spin-X® centrifuge tube filter
15. Centrifuge filter tube for 10 minutes at 2000 rpm
16. Transfer filter solution into autosampler vial

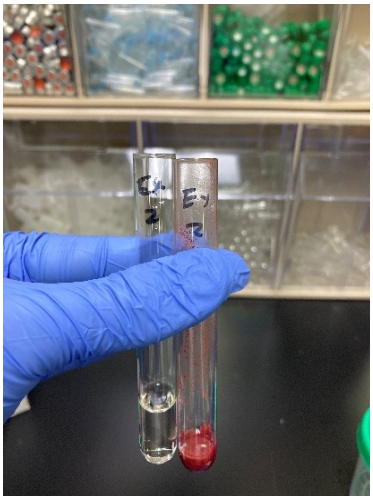


Addition of formate buffer (pH 3)

Addition of acetone



Centrifuge after protein crash



Transferring top layer

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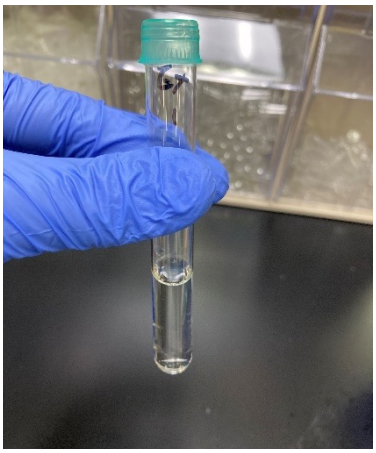
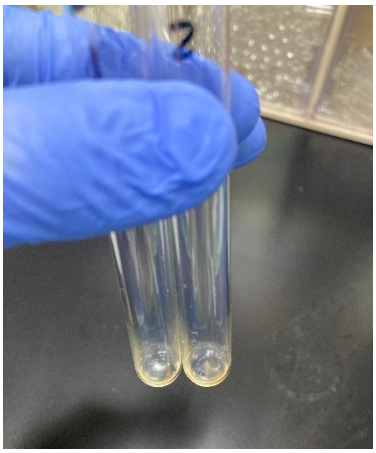
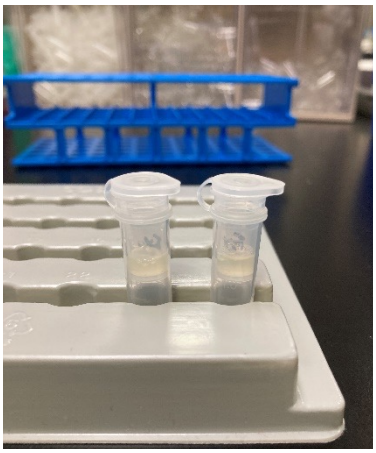


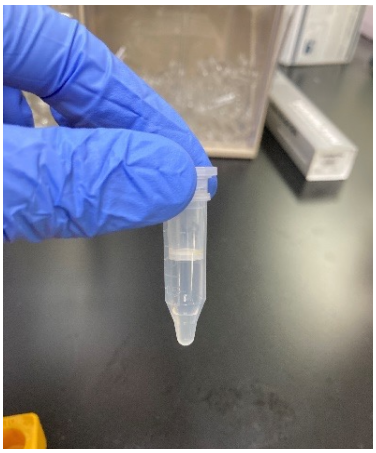
Addition of n-butyl chloride

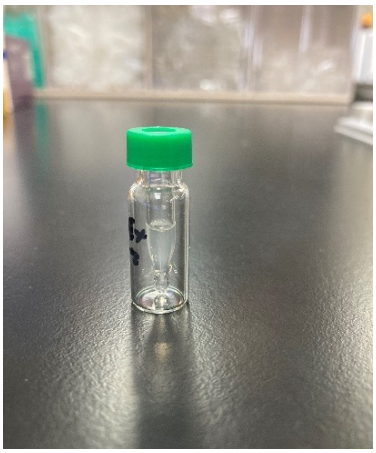


Reconstitute with initial mobile phase condition 95:5 MPA:MPB



Transfer to centrifuge filter tube





Filtered sample solution and transfer to a LC auto-sampler vial with glass insert

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