Document Title:	**Identification of Anticoagulant Adulterants in Seized Material and Biological Samples**
Author(s):	**Barry Logan, PhD, F-ABFT, Taís Regina Fiorentin, Ph.D.**
Document Number:	**305946**
Date Received:	**January 2024**
Award Number:	**2019-DU-BX-0020**

GMS Award 2019-DU-BX-0020

Identification of Anticoagulant Adulterants in Seized Material and Biological Samples

Barry Logan, PhD, F-ABFT, Executive Director
Taís Regina Fiorentin, PhD, Consultant
The Center for Forensic Science Research and Education (CFSRE)
215-366-1513
barry.logan@cfsre.org

Project Period: January 1, 2019 – June 30, 2022

Award Amount: $412,100

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Summary of the Project

While the majority of the forensically most relevant emerging, recreational and therapeutic drugs can be detected in routine tests using gas chromatography mass spectrometry (GCMS) and liquid chromatography tandem mass spectrometry (LCMSMS), there are some more esoteric and more challenging, but highly toxic agents that need the attention of forensic laboratories. These may include events such as the use of Novichok A234 in 2 poisonings in England (1) , and the use of VX nerve agent in the killing of Kim Jong-Nam (2) in Kuala Lumpur airport, in Malaysia.

Another category of agents that has recently been identified as a potential threat are the anticoagulants warfarin and the superwarfarin class. These compounds are used in commercial rodenticides, currently are not controlled, or monitored, and can easily be purchased online in bulk in pure form and imported into the United States without restriction. Additionally, these drugs are available in more dilute, but still toxic, amounts in commercial rodent bait products. The major compounds in this drug class are warfarin, coumatetralyl, brodifacoum, bromadiolone, difenacoum, flocoumafen, diphacinone, pindone, chlorophacinone, and difethialone, shown in Figure 1 (Appendix A). When taken in toxic amounts, the chemicals have serious and frightening consequences, including bloody diarrhea (hematochezia), shortness of breath (dyspnea), abdominal distension, and extreme fatigue, a later occurring but very dangerous sign of advanced poisoning (3). Other clinical manifestations of brodifacoum poisoning include vaginal bleeding (4, 5), nosebleeds (6, 7), blood in the urine (6–13), bleeding from the gums (14, 15), gastrointestinal bleeding (7, 8), subcutaneous bleeding (9, 16), spontaneous abortion (5), coughing up blood (15, 17), abdominal pain (14), and intracranial hemorrhage.

The compounds have a long history of involvement in many types of forensic casework, including suicides, homicides, accidental poisonings, deliberate poisonings with denial (Munchausen and Munchausen-by-proxy), and many deliberate animal poisonings/animal cruelty cases. The National Library of Medicine’s TOXNET database lists over forty recent reports of outbreaks of anticoagulant poisonings in humans (18). There are also multiple media reports from around the world of homicidal poisonings and attempted poisonings involving anticoagulants (19–22). Anticoagulants have also been identified as potential chemical warfare or chemical terrorism agents (23, 24), due to their dramatic effects, the toxicity of the drug, their very long half-lives, the difficulty of diagnosing and treating the patients, and the difficulty of detecting the substances in routine forensic and clinical toxicology and seized drug analysis.

Most significantly, in recent years, these anticoagulants have also been implicated as toxic adulterants in street drugs in the United States, causing significant adverse outcomes and deaths. From a legal point of view, these deaths would be considered homicides resulting from delivery of a toxic substance (23). Recently in 2018 Boyack et al.(25) and Panigrahi et al. (26) reported a large number of cases of synthetic cannabinoids laced with brodifacoum. In March and April 2018 more than 150 patients presented to hospitals in Illinois in an outbreak of severe illness; all the patients presented with coagulopathy and bleeding, and four patients died from major bleeding events (27, 28). Tests in our partner laboratory using a limited scope liquid chromatography high resolution mass spectrometry (LC-QTOF) method confirmed the presence of the superwarfarins brodifacoum, bromadiolone and difenacoum, in many of these patients, and our own laboratory identified them as adulterants in synthetic cannabinoid products resulting

from this outbreak. As of June 2018, 164 cases of synthetic cannabinoid-associated coagulopathy have been reported to the Illinois Department of Public Health (29). This is not the first time that toxic adulterations of street drugs with anticoagulants have taken place. In 1997 La Rosa et al. (30), reported a case of marijuana mixed with brodifacoum causing coagulopathy in the subject that lasted for more than one year, and in 2001, Waien et al. (31) reported a case of crack cocaine contaminated with brodifacoum, causing severe coagulopathies during the following months. Most recently in December 2021, an outbreak occurred in Florida with reports of at least two deaths and several hospitalizations (32–34).

Despite the significant toxicity of the compounds, their criminal justice implications and potential mass poisonings capabilities, the compounds are typically not detected using the standard test approaches employed by United States crime laboratories. There are no color tests for the compounds, and they degrade on-column in GCMS systems, which consequently lack the sensitivity to detect the compounds in seized material. In typical toxicology workflows, the drugs are not detected by routine GCMS or LCMSMS screens for toxic substances due to poor recoveries in acid/base extraction and instrument sensitivity issues.

Major Goals and Objectives

The main goals of this research including performing a systematic evaluation of current routine approaches to the examination and characterization of anticoagulant-containing materials, including commercial baits used in rodent control and anticoagulant laced/adulterated street drugs, and the development of workflows for screening and confirmation/quantitation of the drugs in toxicological samples. Based on these goals, the four main objectives included the following:

> *Objective 1. Assessment of current method capabilities and development of a workflow for anticoagulant adulterants in seized material.*
>
> *Objective 2. Assessment of current method capabilities and development of a workflow for anticoagulant adulterants in toxicology casework.*
>
> *Objective 3. Implementation and analysis of authentic case samples.*

Research Design, Methods, Analytical and Data Analysis Techniques

Objective 1

1.1 Color Tests

Presumptive color tests are an important step in the criminal investigation process, as they can narrow down the identification of the compounds and lead analysts to the next analytical process for the identification of the unknown. The color test reagents selected for this assay included Cobalt Thiocyanate, Dille-Koppanyi, Duquenois-Levine, Mecke, Marquis, Frohede and Mandelin's. Initially the color tests were evaluated using analytical standards using: first generation of coumarins (coumatetralyl and warfarin), second generation of coumarins (brodifacoum, bromadiolone, difenacoum and flocoumafen), indanedione (chlorophacinone, diphacinone and pindone), and thiochromones (difethialone). Initially, analytical standards of the drugs were analyzed using the battery of color tests. For the standards, 1 drop of the color test

reagent was added to a well on a spot plate, which was followed by 1 drop of the anticoagulant analytical standard to the well. In the event the color test required more than one reagent (e.g., Simon's reagent), a drop of the second reagent was added. Any color change occurring in the first minute was noted and recorded along with any changes occurring between one and five minutes. Results can be found in Table 1.

Table 1. Color test results for analytical standards.

		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	Light pink (→brown)	No
Marquis	Color	No color	No color	No color	No color	No color
	Change observed	No	Yellow	Orange	Orange	No
Frohede	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
Frohede	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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The second evaluation of color tests included the assessment of ten commercial products with various anticoagulant active ingredients. The commercial products selected at random according to their availability and active ingredient (Table 2).

Table 2. Commercial rodenticide products used.

Name	**Brand**	**Active Ingredient**
Ramik Green Nuggets	Neogen	Diphacinone
DryUp Bars	Harris	Diphacinone
Just One Bite II Bar	Farnam	Bromadiolone
Havoc-XT Blok	Neogen	Brodifacoum
Ditrac All-Weather Blox	Bell	Diphacinone
TomCat All-Weather Bait Chunk	Motomco	Diphacinone
Bait Block Peanut Butter	JT Eaton	Diphacinone
d-Con Bait Blocks	d-Con	Diphacinone
Rodentex Multi-feed Bars	Farnam	Diphacinone
Rodex Pelleted Bait-1	Neogen	Warfarin

Prior to testing, the commercial products were initially crushed into a powder form using a mortar and pestle. Following, the same procedure was followed. One drop of color reagent was added, followed by adding the commercial product using a tip full from a micro spatula. Any color change within the first minute was noted and color observations were recorded until five minutes. Color test results for the commercial products can be found in Table 3.

Table 3. Color test results for the commercial products.

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

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Cobalt Thiocyanate	Change observed	No	No	No	No	No
Dille-Koppanyi	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	No	No	No	No	No
Duquenois-Levine	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	No	No	No	No	No
Mecke	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	Brown	Brown	No	Brown	No
Marquis	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	Light Brown	Brown	No	No	No
Frohede	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	Light Brown	Brown	No	Brown	No
Mandelin's	Color	Light Green	Light Blue	Green	Yellow	Blue
	Change observed	Dark Green	Dark Blue	No	No	No

The analytical standards analyzed via color tests included: first generation coumarins (warfarin and coumatetralyl); second generation coumarins (bromadiolone, brodifacoum, difenacoum and flocoumafen); indandiones (chlorophacinone, diphacinone and pindone); and thiochromenones (difethialone). The drug standards from the various classes yielded limited color changes with the various tests (Table 1). When the commercial products were tested, color changes were noted for the various tests, however, these color change results were not consistent with the analytical standard results (Table 3). No color change results for the commercial products were noted for the Dille-Koppanyi and Duquenois-Levine color tests. The inconsistency in results may be attributed to the potential of dyes present in the formulation of commercial products, the difficulties of dissolution of solid commercial products, and/or the low concentration of active ingredients in the commercial products. Based on the results of these evaluations, color tests would not be recommended as an initial first test for the analysis of seized material or recovered commercial products due to the limited probative information they provide.

1.2 HPLC-UV Analysis of Commercial Products

Because many commercial products containing anticoagulant drugs contain dyes or dye mixtures, the purpose of this evaluation was to determine if the dyes or dye mixtures could be used to differentiate between different products to allow scene evidence to be related to reference samples to establish which brand was used for adulteration.

A method was developed utilizing an Agilent 1100 series HPLC coupled with diode-array detector (DAD). Separation was achieved using a Nova-Pak C18 (3.9 x 150 mm, 4 µm) column with 20 mM ammonium acetate buffer with 1% acetic acid (mobile phase A) and high purity acetonitrile (mobile phase B) with a flow rate of 1 mL/min. The column temperature was set to 40^{o}C. The gradient for the method can be found in Table 4. DAD signals were collected at 220, 280, 350, 450, and 650 nm. Samples were analyzed using a UV spectrometer at wavelengths ranging from 250-800 nm.

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Table 4. HPLC gradient settings for the analysis of commercial products.

Time (min)	% MPB
0	5
1	5
1.5	20
3	20
6	40
10	60
13	80
16	90
18	90
21	80
24	60
27	50
30	25
32	20
35	5
40	END

Samples were prepared for analysis (HPLC-DAD and/or UV-VIS spectrometer by first grinding the commercial products into a fine powder. Samples were aliquoted (~1g) into a test tube and 5 mL of solvent was added (Table 5). The tubes were vortexed and subsequently headed at 60^{o}C for 10 minutes. Following, samples were sonicated at 40^{o}C for 15 minutes and then centrifuged at 3800 rpm for 10 minutes. Solutions were filtered using gravity filtration with filter paper into a new test tube. An aliquot of 1 mL was transferred for analysis.

Table 5. Suitable solvents for the analysis of commercial products.

Sample ID	Solvent
Ramik Green	Hexane**
DryUp Bars	1:5:44 EDTA in water:acetonitrile:methanol*
Just One Bite II Bar	50:50 water:methanol in acidic environment
Haroc-XT Blok	Toluene
Ditrac All-Weather Blox	Hexane**
Tomcat All-Weather Bait Chunk	Chloroform**
Bait Block Peanut Butter	Toluene
d-Con Bait Block	Hexane
Rodentex Multi-feed Bars	50:50 water:methanol in acidic environment
Rodex Pelleted Bait-1	Hexane**

*A color solution was developed with the solvent; however, no profile was observed on the HPLC-DAD-UV.
**A color solution was developed with the solvent. Nonpolar samples were run on UV-VIS; however, no profile was observed due to interferences.

The following are the UV-VIS spectrum obtained from HPLC-DAD (Figures 2-3).

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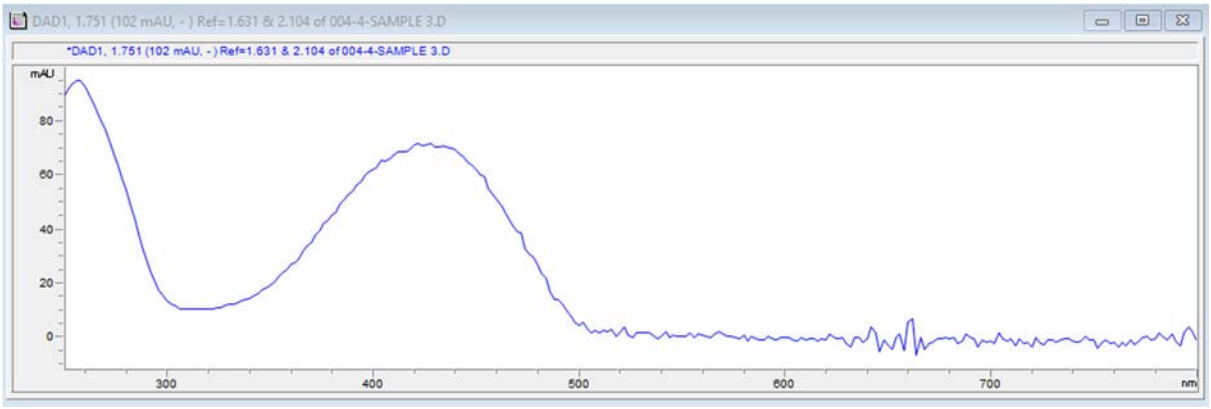

Figure 2-1. Just One Bite II Bar UV-VIS spectrum (280 nm).

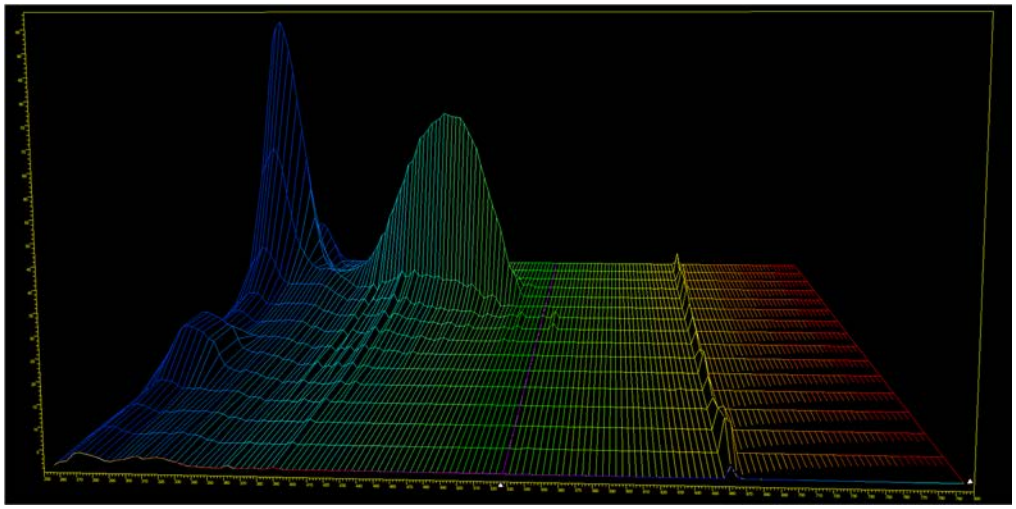

Figure 2-2. Just One Bite II Bar UV-VIS 3D plot.

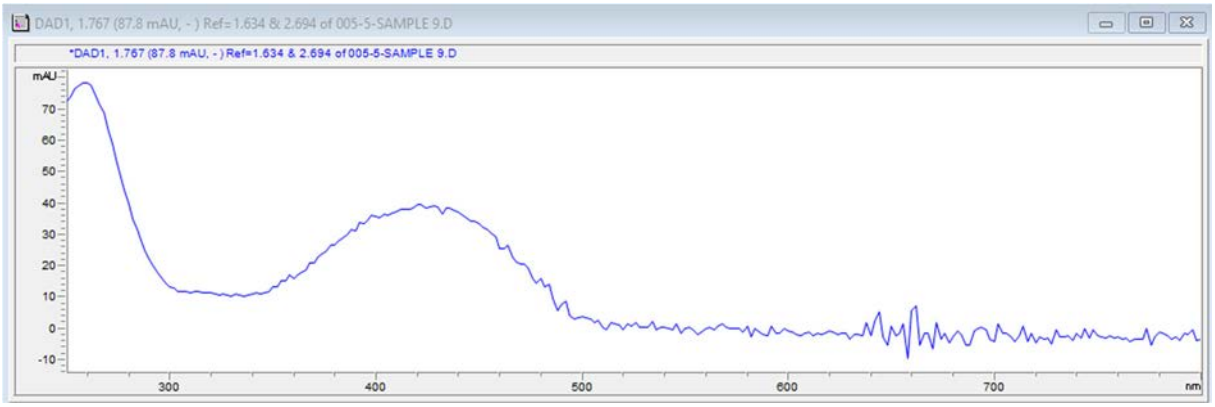

Figure 3-1. Rodentex Multi-feed Bars UV-VIS spectrum (450 nm).

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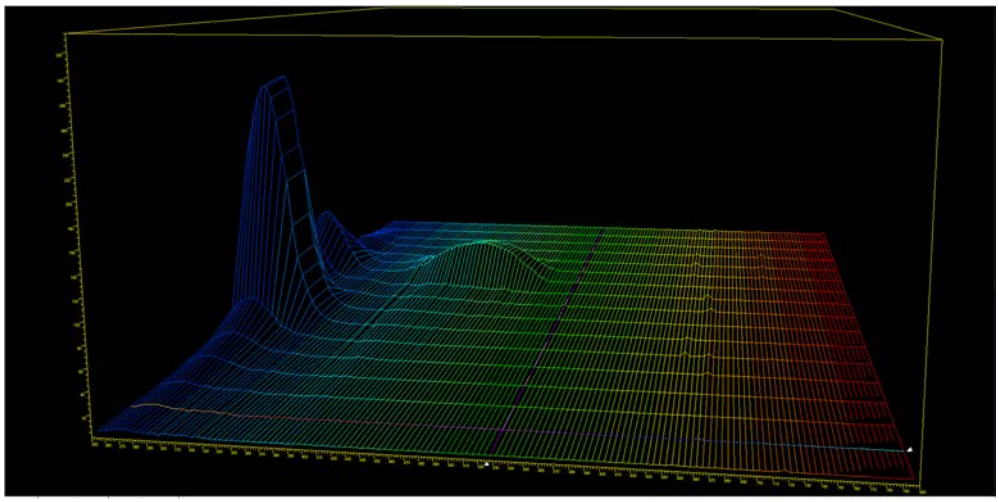

Figure 3-2. Rodentex Multi-feed Bars UV-VIS 3D plot.

Seven commercial rodenticide products (Ramik Green, Haroc-XT Blok, Ditrac All-weather Blox, TomCat All-Weather Bait Chunk, Bait Block Peanut Butter, d-Con Bait Block, and Rodex Pelleted Bait-1) were only soluble in nonpolar solvents (e.g. hexane and toluene). Nonpolar samples are not amenable to analysis using reverse phase HPLC, therefore, these products were analyzed using the UV-VIS spectrometer. The visible spectrums below (Figures 4-6) are dyes from commercial rodenticides (Haroc-XT Blok, Bait Block Peanut Butter, and d-Con Bait Block) products dissolved in nonpolar solvent. Commercial rodenticides (Ramik Green, Ditrac All-weather Blox, TomCat All-weather Bait Chunk, and Rodex Pelleted Bait-1) were dissolved in nonpolar solvent, and the color dyes were visually observed. However, due to limitations in the sensitivity of the instrumentation and interferences in the solvent wash sample, a signal was not obtained for these products.

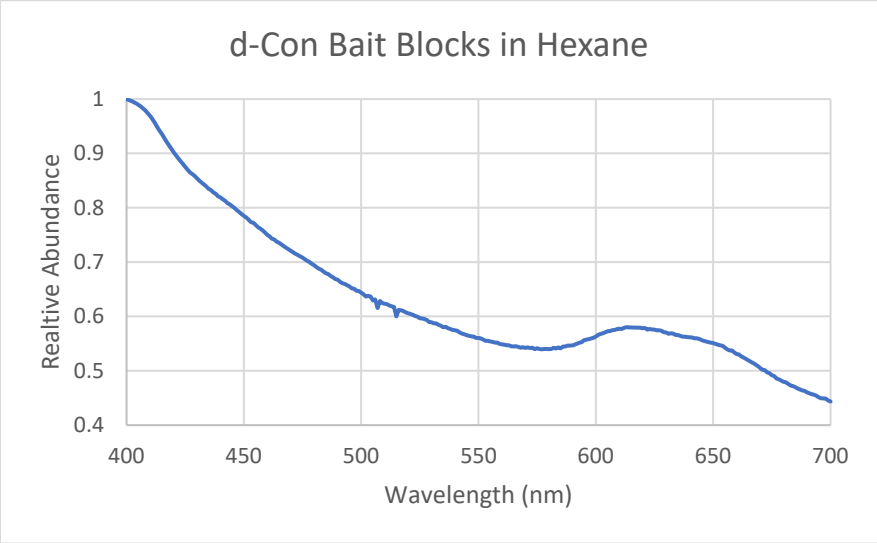

Figure 4. Visible spectrum on UV-VIS for d-CON Bait Blocks.

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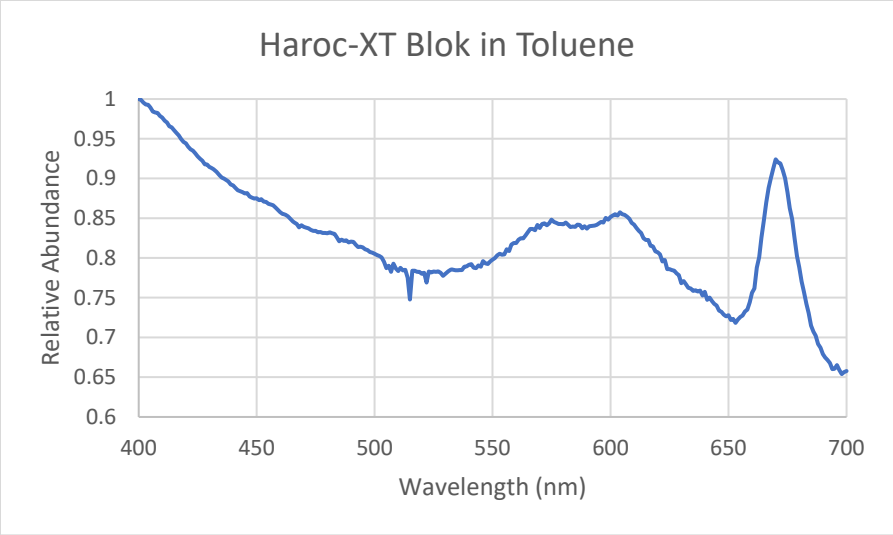

Figure 5. Visible spectrum on UV-VIS for Haroc-XT BLOK.

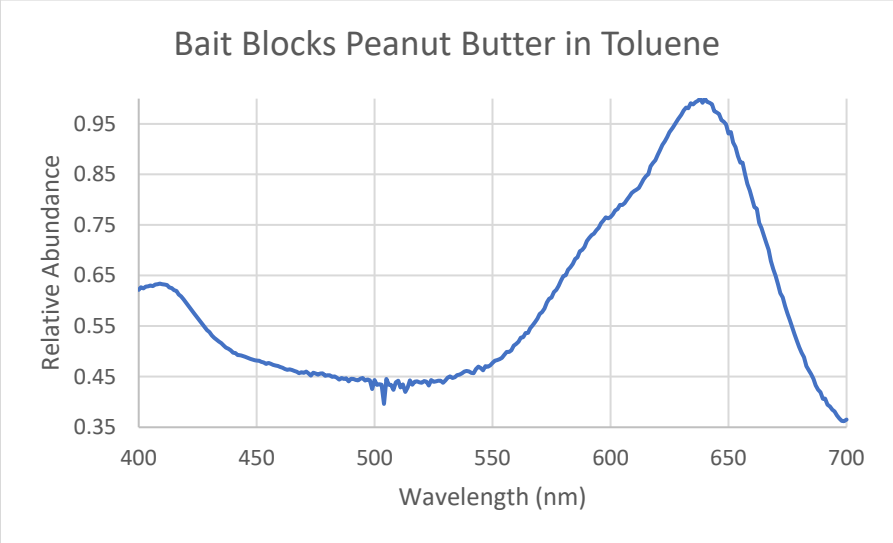

Figure 6. Visible spectrum on UV-VIS for Bait Blocks peanut butter.

1.3. GCMS Analysis and Limit of Detection for Seized Drugs

Method performance characteristics were assessed using an in-house method on an Agilent 6890 gas chromatograph coupled to a 5975 mass spectrometer. Separation was achieved using an Agilent J&W Column DB-1 (0.20 mm x 12 m; 0.33 µm). Helium was used as the carrier gas with a flow rate of 2.3 mL/min. The inlet temperature was set to 265ºC with a 1 µL spitless injection. The initial oven temperature was set to 50ºC and ramped at a rate of 30ºC per minute until 340ºC, and there for hold for 2.33 minutes. The total run time was 12 minutes. The mass spectrometer was set to scan for a mass range of 50-750 m/z. To determine the LOD, the drugs were assessed using serial dilutions of neat standards. The limits of detection (LOD; signal-to-noise ratio greater than 3) for all ten target drugs are provided in Table 6.

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Samples were prepared two different ways, aligning with common practices in forensic chemistry laboratory practices. The ten targeted drugs were prepared by methanolic dilution and by acid-base extraction. For the methanolic dilution, 50 mg of marshmallow leaf was spike with a mix of the target drugs at a concentration of 1 mg/mL. The plant material was allowed to dry overnight at ambient temperature. Once dry, 1 mL of methanol was added along with 200 µL of internal standard (n-propylamphetamine and 10,11-dihydrodibenz[b,f]1,4-oxazepin-11-one 0.5 mg/mL). The samples were vortex, and the liquid was transferred to an autosampler vial for analysis. For the acid-base extraction, the samples were prepared the same way. Following drying, 1 mL of deionized water was added along with 200 µL of internal standard (same as above). Three to five drops of concentrated hydrochloric acid were added and using pH paper the sample was tested to ensure it was acidic. Next, 1 mL of 90:10 dichloromethane and isopropyl alcohol (DCM:IPA) were added. Samples were vortex mixed with the organic layer being transferred to a new test tube. To the original test tube, three to five drops of concentrated ammonium hydroxide were added, and the basicity was checked using pH paper. One mL of 90:10 DCM:IPA was added, followed by vortex mixing and transferring the organic layer into the test tube containing the organic layer from the acidic extraction. One mL of solvent was transferred to an autosampler vial for analysis.

Table 6. Limits of detection for anticoagulant drugs identified via GC/MS.

Drug	**LOD (mg/mL)**	**Identified via Methanolic Dilution**	**Identified via Acid-Base Extraction**
Brodifacoum**	0.1	Y**	Y**
Bromadiolone*	0.2	N	Y*
Chlorophacinone	0.1	N	N
Coumatetralyl	0.01	Y	Y
Difenacoum*	0.2	Y*	Y*
Difethialone**	0.05	Y**	Y**
Diphacinone	0.04	N	Y
Flocoumafen*	0.1	Y*	Y*
Pindone	0.0025	Y	Y
Warfarin	0.02	N	Y

*Identified by the degradation product.
**Both analytes yield the same degradation product, brodifacoum and difethialone were run individually for diagnostic purposes.

Using GC/MS analysis, several anticoagulant drugs such as brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen breakdown into degradation products because of the hot temperature of the injection port. In an attempt to stabilize the drugs and prevent thermal degradation, the five drugs were derivatized using hydroxylamine hydrochloride, BSTFA 1% TMCS and BSTFA 1% TMCS mixed with solvents. However, derivatization did not improve any issues related to degradation.

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The unique degradation products would be use for the detection and identification of the mentioned anticoagulant analytes above. Brodifacoum and difethialone yield the same degradation product, brodifacoum and difethialone were run individually for diagnostic purposes.

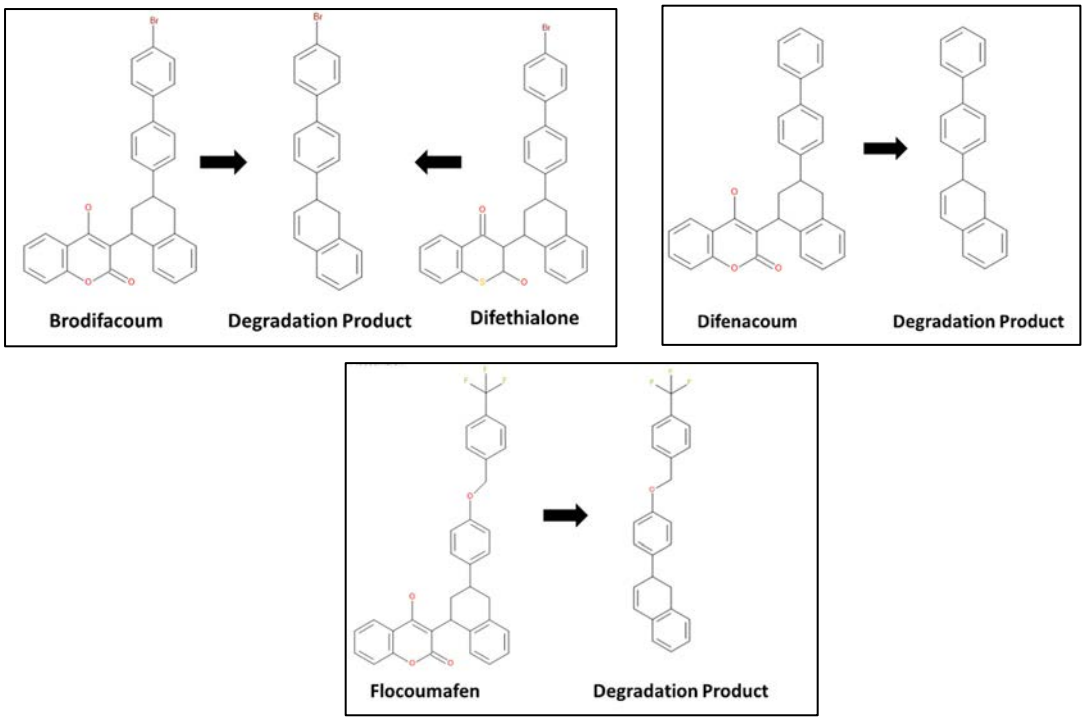

Figure 7. Degradation products. a) Common degradation product for brodifacoum and difethialone. b) Degradation product for difenacoum (unique). c) Degradation product for flocoumafen (unique).

1.4 Optimization and Validation of LCTRAP Screening Method for Seized Material

A method was developed using a Thermo Fisher Vanquish UHPLC system coupled to a QExactive Hybrid Quadrupole-Orbitrap Mass Spectrometer. Chromatographic separation was achieved using on an Accucore™ C18 2.6 μm, 2.1x100mm with 0.1% Formic Acid in Water (MPA) and 0.1% Formic Acid in Acetonitrile (MPB) with a flow rate of 0.3 mL/minute. The column temperature was held at 40ºC. The temperature for the autosampler was set to 10ºC. The gradient for the method can be found in Table 7.

Table 7. UHPLC gradient for the analysis of seized material.

Time (min)	**% MPB**
0	5
1.00	5
3.00	60
7.00	95
7.01	5
8.5	5

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With respect to the mass spectrometer parameters, the instrument operated in positive ionization for all drugs with the exception of bromadiolone. Fragmentation was achieved using stepped collision energy at 20, 40 and 80 eV.

Table 8. MS precursor and fragment ions for 10 anticoagulant drugs.

Drug (Polarity)	Precursor Ion (m/z)	Fragment Ions (m/z)
Brodifacoum (+)	523.09033	335.042, 256.1245, 178.0777, 165.070, 91.055
Bromadiolone (-)	525.0707	283.042, 250.06, 163.02, 93.0300, 78.9200
Chlorophacinone (+)	375.0783	321.0907, 263.0700, 235.0750, 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, 291.101, 159.042, 109.0451
Pindone (+)	231.1016	213.0909, 185.096, 165.0699, 152.0619, 128.062
Warfarin (+)	309.1121	251.07, 191.0337, 163.039, 147.0800, 121.0286

A fit-for-purpose validation was performed following the United Nations Office on Drugs and Crime recommendations (2009) and Scientific Working Group for the Analysis of Seized Drugs (SGWDRUG) Recommendations (2016) (35, 36). Precision, limit of detection, carryover, specificity and autosampler stability were evaluated.

Precision was assessed analyzing 5 replicates of spiked controls at one stipulated concentration (1,000 ng/mL) over three days. Acceptable precision was no more than 20% of the samples should give a false negative result. The criteria for positive results included: retention time within ± 30 seconds, mass error within 20 ppm, fit threshold of 80% for isotopic pattern, fragment ions within 50 ppm (minimum of 1 fragment), and library score greater than 50. The limit of detection concentration was administratively set and evaluated at 100 ng/mL in 10 replicates over three days. Criteria for identification required the above parameter as well as a signal to noise ratio greater than three. Assay interference was assessed by analyzing neat reference solutions of the potential interfering substances frequently encountered in seized drugs including common drugs (cocaine, fentanyl and methamphetamine) and adulterants (caffeine, lidocaine, procaine, benzocaine, phenacetin, metamizole, diphenhydramine, aminopyrine, diltiazem, hydroxyzine, levamisole, strychnine and xylazine). Carryover was determined by injecting three different concentrations (2,000, 10,000 and 100,000 ng/mL) followed by blank controls. The highest concentration at which no analyte carryover was observed in the blank control was determined to be the concentration at which the method was free from carryover.

Autosampler stability was performed analyzing samples immediately after preparation to establish the time zero responses, the following sets were analyzed after 24, 48 and 72 hours stored refrigerated on the autosampler at 10°C. The analytes were considered stable when identified following the positivity criteria described above.

Samples were prepared by methanol extraction of seized plant material. Fifty (50) mg of plant material was spiked with target drugs, followed by the addition of 1 mL of methanol. The sample was vortex mixed and then further diluted to 1:99 with mobile phase A:B 95:5 followed by the addition of 50 µL of internal standard IS (Brodifacoum-d4, 20 ng/µL). The method was successfully validated for all 10 drugs. The method was free from carryover up to 10,000 ng/mL for chlorophacinone, difenacoum, and diphacinone and 100,000 ng/mL for the remaining compounds. The method was free from interferences from commonly encountered drugs and adulterants. All drugs 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.

Objective 3

3.1 Implementation of Developed Methods for Authentic Seized Material

The Pinellas County Forensic Laboratory (Pinellas County, Florida) provided 50 samples of suspected synthetic cannabinoid drug extracts for analysis. The samples were diluted with 1 mL of methanol and analyzed using the developed method on the GC/MS. No anticoagulant adulterants were identified in the synthetic cannabinoid samples.

Objective 2

2.1. Assessment of Current Method Capabilities for Testing

This section includes an assessment of GCMS and LCQTOF screening methods, as well as review of the literature on the published methods. The results of the literature review are shown in Table 9.

Table 9. Review of published literature methods for anticoagulant drugs.

Drugs	Technique	Matrix	Assay range LOD	Sample Preparation	Column Mobile Phase	Reference
Warfarin, Coumachlor, Coumatetralyl, Bromadiolone	GC-MS	-	No range provided LOD: Warfarin and coumachlor 20 ng/mL, coumatetralyl 10 ng/mL, bromadiolone 30 ng/mL	SLE using Oasis HLB cartridge and eluted with chloroform:isopropanol (9:1).	DB-5MS (30m x 0.25mm, 0.25µm)	S. Sato, Coumarin rodenticides, in: Drugs and Poisons in Humans, Springer, Berlin Heidelberg, 2005, pp. 599–608.
Brodifacoum, Bromadiolone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen	LC-MS/MS	Dog blood and feces	Brodifacoum 2.6-1309 ng/mL Bromadiolone 2.6-1319 ng/mL Coumatetralyl 1.5-731 ng/mL Difenacoum 2.2-1111 ng/mL Difethialone 2.7-1349 ng/mL Flocoumafen 2.7-1356 ng/mL LOD: 1.5-2.7 ng/mL	PPT with ACN followed by LLE with ethyl acetate:heptane (4:1).	Waters Acquity UPLC BEH C18 (2.1x50mm, 1.7µm) 5mM ammonium formate (pH 10.2) and MeOH	Seljetun, K. O. et al., Quantitative method for analysis of six anticoagulant rodenticides in feces, applied in a case with repeated samples from a dog. Acta veterinaria scandinavica, 60(1), 3. 2018
Brodifacoum (cis- and trans-), Difenacoum (cis- and trans-), Bromadiolone	LC-MS/MS	Human plasma	No range provided LOD: 0.125-0.63 ng/mL	PPT with CAN.	Aglient Poroshell EC-C8 (50x2.1mm, 2.7µm) 0.01%FA in Water and 0.01% FA in ACN	Feinstein, D. L. et al., Effects of vitamin K1 treatment on plasma concentrations of long-acting anticoagulant rodenticide enantiomers following inhalation of contaminated synthetic cannabinoids. Clinical Toxicology, 1-9. 2019
Brodifacoum, Bromadiolone, Coumachlor, Coumatetralyl,	LC-MS/MS	Human Blood	1-2000 ng/mL LOD: 1 ng/mL	PPT with 0.1% FA in ACN followed by passing supernatant through Phree	Waters Acquity UPLC BEH C18 (2.1x100mm, 1.7 µm) 0.1% FA and 100mM	Guo, H. et al., Sensitive and simultaneous determination of

Difenacoum, Warfarin, Pindone, Diphacinone, Chlorophacinone				Phospholipid removal cartridge.	ammonium formate and ACN	nine anticoagulant rodenticides in human blood by UPLC–MS-MS with phospholipid removal pretreatment. Journal of analytical toxicology, 42(7), 459-466. 2018
Pindone, Coumatetralyl, Warfarin, Coumachlor, Difenacoum, Brodifacoum, Bromadiolone, Difethialone, Flocoumafen	LC-MS/MS	Human Blood	Coumatetralyl, warfarin, and coumachlor 0.02-200 ng/mL Pindone, difenacoum, brodifacoum, difethialone and flocoumafen 0.07-200 ng/mL LOD: 0.02-0.07 ng/mL	SLE using Biotage Isolute. Sample pretreated with citric acid/sodium hydroxide buffer (pH 6). Eluted with ethyl acetate.	Acquity UPLC BEH T3 (2.1x100mm, 1.7 µm) 0.1% FA in Water and 0.1% FA in MeOH	Gao, X. et al., Sensitive determination of nine anticoagulant rodenticides in blood by high resolution mass spectrometry with supported liquid extraction pretreatment. Forensic science international, 292, 39-44. 2018
Warfarin, Coumatetralyl, Chlorophacinone, Bromadiolone, Difenacoum, Brodifacoum, Flocoumafen, Difethialone	LC-MS/MS	Rat Liver	Coumatetralyl, chlorophacinone, bromadiolone 2-1000 ng/mL Warfarin 1-1000 ng/mL Difenacoum, brodifacoum, flocoumafen 3-1000 ng/mL Difethialone 4-1000 ng/mL LOD: 0.92-2.2 ng/g	SLE after homogenization with 10 mL of acetone using UltraTurax.	Poroshell 120 Stable Bond C18 10mM Ammonium Acetate (pH 5.7) and ACN	Fourel, I. et al., Core-shell LC–MS/MS method for quantification of second generation anticoagulant rodenticides diastereoisomers in rat liver in relationship with exposure of wild rats. Journal of Chromatography B, 1041, 120-132. 2017
Acenocoumarol, Coumachlor, Coumatetralyl,	LC-MS/MS	Serum	5 – 250 ng/mL LOD: 5 ng/mL	LLE using pH 4.2 potassium buffer and acetone.	Waters Atlantis C18 (2.1x20mm, 3 µm)	Grobosch, T. et al., Acute bromadiolone intoxication. Journal

Phenprocoumon, Warfarin, Brodifacoum, Bromadiolone, Difenacoum, Difethialone, Flocoumafen					MeOH and 0.1% FA in Water (10:90)	of analytical toxicology, 30(4), 281-286. 2006
Bromadiolone, Brodifacoum, Difenacoum, Chlorophacinone, Diphacinone, Coumachlor, Warfarin	LC-MS/MS	Liver	Bromadiolone, difenacoum, chlorophacinone, diphacinone, cloumachor, warfarin 0.1 - 150 ug/kg Brodifacoum 100 - 300 ug/kg LOD: 0.1 - 0.5 ug/kg	QuEChERS with CAN.	Thermo Hypersil Gold aQ C18 (100 mm x 2.1 mm, 1.9 µm) 4mM ammonium formate w/ 0.1% FA in water and 4mM ammonium formate w/ 0.1% FA in MeOH	López-García, M. et al., Determination of rodenticides and related metabolites in rabbit liver and biological matrices by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 137, 235-242. 2017

2.2 Assessment of Current Toxicology Workflows

LC-QTOF Screening

Samples were prepared in blood at concentrations of 15, 100 and 200 ng/mL using mixes containing all ten target drugs and extracted using our in-house screening protocol to characterize the performance and limitations of this method. Samples were extracted using a simple, single-step liquid-liquid extraction (LLE). Samples were aliquoted (0.5 mL) for analysis and 50 µL (0.1 ng/µL) of internal standard (Brodifacoum-d4) was added. Samples were basified using 1 mL of 0.1 M borax buffer (pH 10.4) and extracted into 3 mL of N-butyl chloride/ethyl acetate (70:30). The resulting samples were roto-mixed for 10 minutes, followed centrifugation at 4500 rpm for 15 minutes. The supernatant was then transferred and dried to completion at 35˚C under air (10 psi). Samples were reconstituted in 200 µL of LC initial conditions (95:5 A:B). Samples were analyzed screened using a Sciex TripleTOF® 5600+ quadrupole time-of-flight mass spectrometer coupled to a Shimadzu Nexera ultra high-performance liquid chromatograph (Shimadzu, Kyoto, Japan). A reverse phase gradient of ammonium formate (10mM, pH 3) and methanol/acetonitrile (50:50) was used to create chromatographic separation on a Phenomenex® Kinetex C18 analytical column (50mm x 3.0mm, 2.6µm) at a flow rate of 0.4 mL min^{-1}, for a total run time of 15.5 minutes. The injection volume was 10 µL. Following positive electrospray ionization, precursor ions were acquired by TOF MS scan and isolated based on overlapping mass range windows (SWATH™ acquisition). Fragmentation was achieved using a rolling collision energy of 35±15eV. Data processing was performed using PeakView (Version 2.2) and MasterView™ (Version 1.1) software.

At 200 ng/mL, the method was able to detect and identify brodifacoum, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin. Bromadiolone and diphacinone were also detected at this concentration, however, bromadiolone failed to meet retention time criteria and diphacinone failed the minimum library score criteria. At 100 ng/mL, brodifacoum, coumatetralyl, difenacoum, difethialone, flocoumafen and warfarin were identified. At 15 ng/mL, coumatetralyl, difenacoum, flocoumafen and warfarin were detected. Chlorophacinone and pindone were not detected at any of the three concentrations levels mentioned above, therefore, a targeted extraction and method may be necessary should those drugs be suspected in toxicology casework.

GC-MS Screening

Samples were sent to a commercial laboratory for analysis to evaluate existing workflows and their ability to detect anticoagulant drugs in biological samples. Three samples containing at 10 anticoagulant drugs were prepared at 15 ng/mL, 100 ng/mL and 200 ng/mL and submitted blind to the laboratory. Samples were analyzed using a general basic screen followed by analysis on a gas chromatograph mass spectrometer. In this workflow, none of samples returned a positive screening result. An additional three samples containing all 10 drugs at the concentrations noted above were submitted for qualitative analysis on a targeted anticoagulant panel. The panel contained brodifacoum, bromadiolone, chlorophacinone, dicumarol (not included in spiking mixes), difenacoum, diphacinone and warfarin with analysis by liquid chromatography tandem mass spectrometry. At 15 ng/mL, chlorophacinone diphacinone and warfarin were detected. At 100 and 200 ng/mL, all drugs were detected.

2.3 Development and Validation of LCTRAP Screening Method for Toxicology Samples

The method develop for the analysis of seized material (Section 1.5) was modified for the analysis of toxicological samples using a Thermo Fisher Vanquish UHPLC system coupled to a QExactive Hybrid Quadrupole-Orbitrap Mass Spectrometer. Chromatographic separation was achieved using on an Accucore™ C18 2.6 µm, 2.1x100mm with 0.1% Formic Acid in Water (MPA) and 0.1% Formic Acid in Acetonitrile (MPB) with a flow rate of 0.3 mL/minute. The gradient for the method can be found in Table 7. The column temperature was held at 40ºC. The temperature for the autosampler was set to 10ºC. The mass spectrometer conditions were also identical to those used for the analysis of seized material (Section 1.5). The MS precursor and fragment ions can be found in Table 8.

For sample extraction, 0.5 mL of blood was used followed by the addition of 25 µL of internal standard (Brodficoum-d4). Following 0.5 mL of formate buffer (pH 3) was added along with 2 mL of acetone. Samples were capped and rotated for five minutes followed by centrifugation at 3000 rpm for five minutes. The supernatant was transferred to a new test tube were 1.5 mL of n-butyl chloride was added. Samples were again capped and rotated for five minutes and centrifuged at 3000 rpm for five minutes. The supernatant was transferred to a new tube and dried to completion at 36ºC. Samples were reconstituted in 100 µL of mobile phase (95A:5B).

A qualitative validation was performed assessing the limit of detection (LOD), precision, carryover, interference, processed sample stability (up to 72 hours), and interference. Blank human whole blood was used for the assessment. For the LOD (administratively set at 100 ng/mL), samples were analyzed in triplicate over the course of five days. Precision was assessed at 200 ng/mL by analyzing samples in duplicate over the course of five days. Carryover was assessed by preparing samples at 1000 ng/mL and 2000 ng/mL and subsequently analyzing two extracted blanks following each concentration. For processed sample stability, all drugs were prepared in human blood at 200 ng/mL in triplicate. Samples were analyzed at 0, 24, 48 and 72 hours. Interference from the matrix, internal standard and commonly encountered drugs was assessed, including synthetic cannabinoids. Older generation synthetic cannabinoids were spiked into human whole blood at a concentration of 200 ng/mL (UR-144, XLR-11, AB-PINACA, ADBICA, ADB-PINACA, PB-22, AM-2201, AKB-48, JWH-201 and 5F-PB-22). Additionally, samples were prepared with the target 10 anticoagulant drugs and synthetic cannabinoids (MDMB-4en-PINACA, 4-cyano-MDMB BUTINACA, 4F-MDMB-BINACA, 4F-MDMB 3,3-Dimethylbutanoicacid, 4-fluoro ABUTINACA and 5-fluoro EDMB-PINACA).

Acceptability criteria included all analytes meeting reporting criteria for positive results (retention time ±0.25, ppm error <5, at least 1 fragment identified and signal to noise greater than 3:1 for the LOD). Samples were considered stable if they met positive identification reporting criteria and the response was not more than a 20% loss from day 0. All 10 target drugs (brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, diphacinone, flocoumafen, pindone and warfarin) met acceptability criteria at the LOD of 100 ng/mL and met precision acceptability criteria at 200 ng/mL. The method was free from carryover up to concentrations of 2000 ng/mL. All drugs were stable up to 72 hours. No interferences were identified from the matrix or internal standard. No interferents were identified from the older generation synthetic cannabinoids. With the newer generation synthetic cannabinoids, difethialone and diphacinone were not identified.

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2.3. Development of an LCMSMS Quantitative Method for Toxicological Confirmations

A method was developed and validated for biological samples using a Waters Acquity UPLC coupled to a Waters Xevo TQ-S Micro triple quadrupole mass spectrometer. Chromatographic separation (Figure 8) was achieved using on an Acquity UPLC BEH C18 1.7µm, 2.1x100mm with 0.02% Ammonium Hydroxide in Water (MPA) and 0.02% Ammonium Hydroxide in Methanol (MPB) with a flow rate of 0.3 mL/minute with a 10 µL injection volume. The column temperature was held at 60ºC. The temperature for the autosampler was set to 15ºC. The gradient for the method can be found in Table 10.

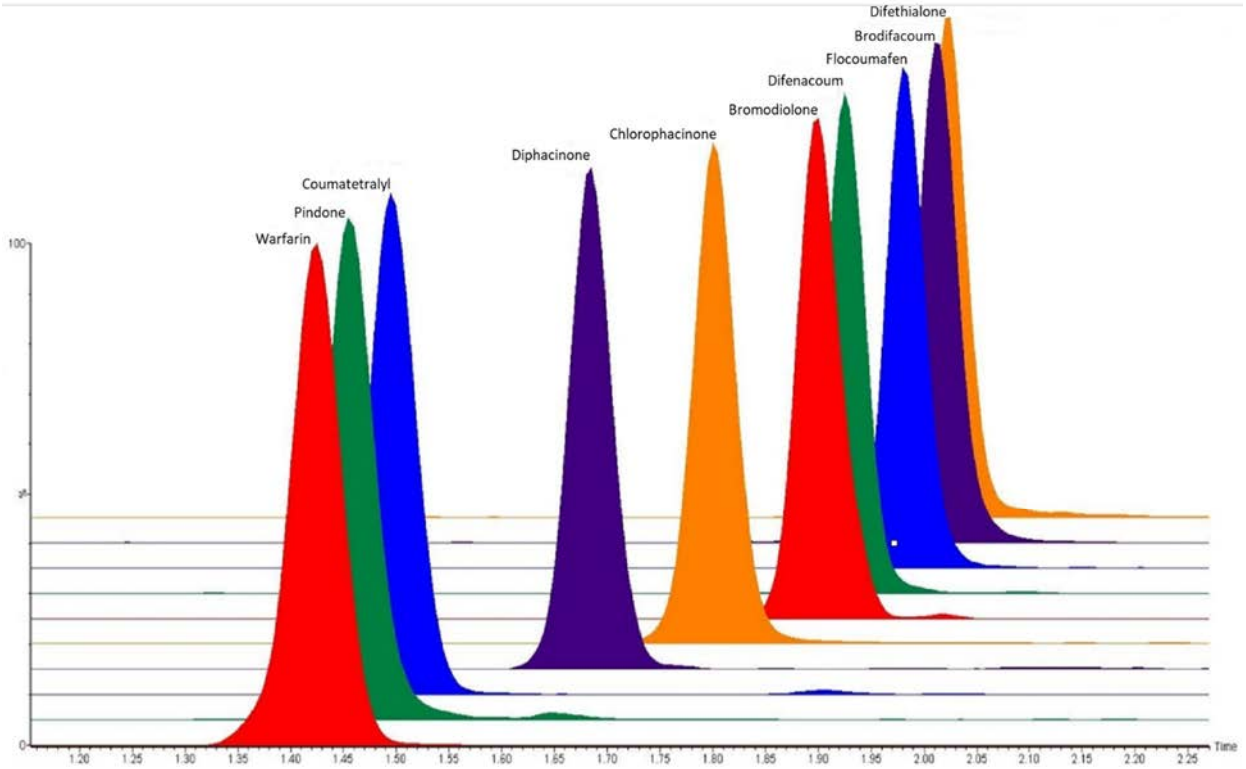

Figure 8. Total ion chromatogram of a 50 ng/mL extracted calibrator.

Table 10. UPLC gradient for the analysis of seized material.

Time (min)	**% MPB**
Initial	5
1.60	95
3.50	95
3.60	5
4.00	5

With respect to the mass spectrometer parameters, the instrument operated in negative ionization for all drugs. MRM transitions, cone voltage and collision energy for all target analytes can be found in Table 11. Deuterated forms of the targeted drugs were purchased and used as internal standard for the analysis.

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Table 11. MS parameters and transitions.

Drug (Retention Time, mins)	Precursor Ion to Quantification Ion (m/z) Precursor Ion to Qualifier Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
Brodifacoum (2.17)	521.20 → 135.10 523.20 → 135.10	42 55	40 38
Brodifacoum D4	525.30 → 139.10 527.30 → 139.10	42 55	42 38
Bromadiolone (2.04)	525.30 → 250.10 525.30 → 181.10	42 42	38 36
Bromadiolone D5	530.30 → 255.20 530.30 → 181.10	42 42	36 34
Chlorophacinone (1.93)	373.20 → 201.10 373.20 → 145.10	12 12	22 20
Chlorophacinone D4	377.20 → 201.10 377.20 → 149.10	12 12	22 22
Coumatetralyl (1.56)	291.20 → 141.10 291.20 → 106.10	42 42	26 26
Coumatetralyl D4	295.20 → 141.10 295.20 → 110.10	42 42	26 28
Difenacoum (2.08)	443.30 → 135.10 443.30 → 293.20	42 42	34 32
Difenacoum D4	447.30 → 293.20 447.30 → 139.10	42 42	32 36
Difethialone* (2.19)	537.20 → 79.00 537.20 → 151.10	42 42	46 38
Diphacinone (1.78)	339.30 → 167.10 339.30 → 116.10	30 30	22 44
Diphacinone D4	343.20 → 167.10 343.20 → 120.10	30 30	24 42
Flocoumafen (2.15)	541.40 → 382.20 541.40 → 161.10	42 42	24 34
Flocoumafen D4	545.40 → 386.20 545.40 → 165.10	42 42	26 36
Pindone** (1.51)	229.10 → 116.10 229.10 → 172.10	28 28	35 20
Warfarin (1.48)	307.20 → 161.10 307.20 → 250.20	30 30	20 22
Warfarin D5	312.20 → 161.10 312.20 → 255.20	30 30	20 22

*Difethialone uses brodifacoum D4 for internal standard, **Pindone uses Warfarin D5 for internal standard

An LLE method was developed for the analysis using 0.5 mL of blood. After aliquoting 0.5 mL of blood, 25 µL of internal standard (1 ng/µL) was added along with 0.5 mL of ammonium formate (pH 3) and 2 mL of acetone. Samples were capped and rotated for five minutes followed by centrifugation at 3000 rpm for five minutes. The supernatant was transferred to a new test tube where 1.5 mL of n-butyl chloride was added. Samples were again capped and rotated for five minutes followed by centrifugation at 3000 rpm for five minutes. The supernatant was dried to completion at 35ºC and reconstituted in 200 µL of 95:5 MPA:MPB. Samples were subsequently vortexed and transferred to a Costar® Spin-X® centrifuge tube filter and centrifuged at 7000 rpm for 10 minutes. The supernatant was transferred to an autosampler vial for analysis.

Quantitative method validation was based on the standards set by Academy Standards Board in Forensic Toxicology (ANSI/ASB Standard 036) (37). Parameters evaluated included calibration model, bias and precision, limit of quantification (LOQ), limit of detection (LOD), carryover, interferences, matrix effects and process efficiency. Additional parameters evaluated included recovery, auto-sampler stability, and matrix matching.

The calibration range for bromadiolone, brodifacoum, and pindone was 50 – 500 ng/mL and had a resulting limit of quantification and detection at 50 ng/mL. The limit of quantification and detection for chlorophacinone, coumatetralyl, difenacoum, diphacinone, flocoumafen, and warfarin was 10 ng/mL and had a calibration range of 10 – 300 ng/mL. Difethialone did not meet acceptability requirements for quantitative identification and therefore was identified qualitatively at a cutoff concentration of 80 ng/mL. All calibration curves were quadratic using 1/x weighting regression analysis of the peak area of the analyte to the peak area of the internal standard.

The bias for compounds using the calibration range of 50-500 are the following: <15.2% for the low control (80 ng/mL), <3.2% for the middle control (240 ng/mL) and <9.4% for the high control (240 ng/mL). The bias for compounds using the calibration range of 10-300 ng/mL were <4.4%, <4.4% and <2.1%, for the low (20 ng/mL), middle (240 ng/mL) and high control (240 ng/mL), respectively. Within and between run precision met acceptability criteria for percent coefficient of variation and accuracy for all ten drugs. The method was free from carryover up to 500 ng/mL.

Recovery ranged from 35% to 77% across all compounds. Coumatetralyl show the lowest recovery at 35%, however, even with a low recovery with the defined extraction, the compound was still able to be accurately quantitated at 10 ng/mL. Matrix effects and process efficiency were also evaluated. It was determined that the matrix had a large effect on this method. Because these compounds are anticoagulants, blood received for cases was thin and without clots. If our in-house antemortem blood was not fresh, an effect was seen on the data. However, the effects were mitigated for anticoagulants that had a paired deuterated internal standard.

No interferences were identified from the matrix, internal standards, or commonly encountered drugs, including cocaine, fentanyl, methamphetamine, aminopyrine, benzocaine, caffeine, levamisole, lidocaine, phenacetin, procaine, strychnine, diphenhydramine, ADB-FUBICA, ADB-HEXINACA, 4-cyano-MDMB BUTINACA, 4F-ABUTINACA, 4F-MDMB-BINACA, MDMB 3,3-Dimethylbutanaic acid, 5-chloro MDMB-PICA, 5F-EDMB-PINACA, 5F-EMB-

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PICA, 5F-MDMB-PICA, MDMC-4en-PINACA, U-47700 and U-47931E. All drugs were stable at 15ºC for 72 hours.

The validated method was assessed to determine the usability of alternative matrix types. Serum was prepared at low and high concentrations (20/80 and 240/460 ng/mL, respectively). Samples were extracted and analyzed using a calibration curve prepared in blood on the same day. The method was successfully matrix matched if the target drugs met positive reporting criteria and the mean was ±20% of target concentration and %CV <20%. All compounds, except for pindone, met acceptability criteria of matrix matching to serum/plasma.

Objective 3

3.1 Implementation of Developed Methods for Authentic Biological Samples

Authentic deidentified cases (n=79) from the December 2021 anticoagulant outbreak in Florida were acquired. The samples were extracted using the extraction protocol and analyzed using the quantitative methodology developed and validated in Objective 2.3. In 74 samples, brodifacoum was detected and quantitated across three different matrices. In six serum samples, difenacoum was detected and reported qualitatively. Results are shown below in Table 12.

Table 12. Concentration results from authentic specimen testing.

Drug	Matrix	Mean Concentration (ng/mL)	Median Concentration (ng/mL)	Range (ng/mL)
Brodifacoum	Blood (n=13)	159.5(±131)	110	48.8-429.8
	Serum (n=45)	575.9(±432)	476.3	86.2-1995
	Plasma (n=16)	134.3(±133)	53.2	11.5-365.3

The developed method was highly efficient for the extraction of anticoagulant drugs, which are highly lipophilic. The analytical method proved to be sufficiently sensitive and specific for the detection and quantitation of ten anticoagulant drugs in human blood or serum. With respect to authentic cases, brodifacoum was identified in authentic cases in three different matrices. Concentrations were highest in serum samples, but paired samples were not available to compare matrices within individual subjects. Additionally, some serum samples were also positive for difenacoum, which was detected at much lower concentrations relative to brodifacoum. Laboratories should consider including additional anticoagulant drugs in their panels. As the number of incidents involving anticoagulant drugs adulterating seized material increases, laboratories should be aware of the challenges associated with the detection of anticoagulant drugs and consider incorporating these drugs into their panels.

Expected Applicability of the Research

Several challenges associated with the detection of anticoagulants in biological matrices and/or seized material in the context of suspected poisonings related to the two outbreaks that have been described. The chemical properties of the drugs such as large mass, low volatility, polarity, and thermal instability create analytical challenges. Additionally, because these products are being laced onto synthetic cannabinoids as adulterants, the amount of drug is likely to be small, which requires additional analytical sensitivity. Through the work the has been completed as part of this

grant, we have provided an assessment and demonstrated the limitations associated with traditional workflows for both seized drug analysis and toxicological analysis. To address these short comings, we have developed workflows designed to detect these drugs with sufficient sensitivity using a variety of technologies available to crime laboratories within the criminal justice system. All of the developed extraction protocols and analytical method parameters have been disseminated and are available as a resource to crime laboratories. The Superwarfarin Toolkit developed under this award provides validated methods and extraction protocols available for implementation in laboratories for both the analysis of seized material and biological samples, which would eliminate any development time needed should another outbreak occur.

Participants and Other Collaborating Organizations

The work performed under this award would not have been possible without collaboration and partnerships. All laboratory-based assessments and developments were completed at the CFSRE and involved Hiu Yu Lam, Amanda Mohr, Barry Logan, Tais Fiorentin (formerly of CFSRE), Sam Krug and Fran Diamond. Collaborating agencies included Thermo Fischer Scientific (Waltham, MA), Waters Corporation (Milford, MA), and NMS Labs (Horsham, PA). Authentic samples were provided by the Pinellas County Crime Lab (Largo, FL) and NMS Labs.

Outcomes

Activities/Accomplishments

The following is a summary list of the activities and accomplishments that occurred related to the overall objectives of the project.

Objective 1. Assessment of current method capabilities and development of a workflow for anticoagulant adulterants in seized material.

- Reviewed and assessed commonly used seized drug analysis workflows for the detection of anticoagulant drugs including color tests and GC/MS analysis.
- Identified limitations associated with the detection of anticoagulant drugs using GC/MS.
- Developed and validated a method using high resolution mass spectrometry.

Objective 2. Assessment of current method capabilities and development of a workflow for anticoagulant adulterants in toxicology casework.

- Reviewed and assessed commonly used toxicology workflows for the detection of anticoagulant drugs including color tests and GC/MS analysis.
- Provided a review of the literature related for methods developed for the analysis of anticoagulant drugs.
- Developed novel extraction protocols and validated analytical methods for both LC-MS/MS and using high resolution mass spectrometry for the analysis of anticoagulant drugs in biological matrices.

Objective 3. Implementation and analysis of authentic case samples.

- Analysis of 50 authentic seized drug samples for the analysis of anticoagulant drugs from Pinellas County, Florida.
- Analysis of 72 authentic biological samples from the December 2021 anticoagulant drug outbreak in Florida using development methods.

Results and Findings

A comprehensive assessment of the detection of anticoagulant drugs in seized material and biologicals was performed. Based on this assessment, limitations were noted associated with the detection of anticoagulant drugs both for seized material testing and toxicology. Of note, for seized material analysis, one of the commonly used practices for initial screening is the use of color tests. Analytical standards of anticoagulant drugs did produce color changes, however, when tested using commercial products where a particular anticoagulant was listed as the active ingredient, the colors changes noted were inconsistent. With respect to GC/MS analysis, one of the major limitations associated with the detection of anticoagulants (brodifacoum, bromadiolone, difenacoum, difethialone, and flocoumafen). It should be noted that brodifacoum and difethialone yield the same degradation product, therefore, alternative analysis methods would be required. Laboratories should be aware that difenacoum and flocoumafen yield unique degradation products, which could be used for diagnostic purposes. Method characterization, performance limits and alternative methods are fully described under Objective 1 in the Research Design section above.

With respect to toxicological analysis, existing methods using GC/MS did not yield any positive identifications when subject to a routine test. Within a targeted assay, positive results were obtained, but limited by the scope of the method. Using LC-QTOF screening, a broad-based screening approach, proved to be a more effective screening technique, especially at high concentrations (200 ng/mL) for most anticoagulant drugs. At lower concentrations (15 ng/mL) only four of the ten target drugs were positively identified. It should be noted that chlorophacinone and pindone were not detected at any of the three concentrations levels mentioned above, therefore, a targeted extraction and method may be necessary should those drugs be suspected in toxicology casework. Based on these limitations, a targeted extraction technique and analytical method was developed and validated. The method was subsequently implemented provided sufficient sensitivity and selectivity for the analysis of authentic biological samples. Additional information related to performance characteristics and method details are described in Objective 2 in the Research Design section above.

Limitations

While the overarching goals and objectives of the research were met, there are limitations that should be noted. Characterization of existing methods for both seized material and toxicology methods were limited to those used in-house or by our partnering agency. If laboratories use other methods, they will need to independently assess and characterize them. With respect to color tests, authentic samples containing synthetic cannabinoids were not able to be acquired and evaluated, so the performance with authentic samples is unknown. Additionally, the amount of anticoagulant drug lacing the synthetic cannabinoid material could be highly variable and could potentially impact color tests. With respect to toxicology testing, only ten anticoagulants were included in the method.

Artifacts

List of Products

A presentation entitled “Evaluation of Presumptive Color Tests for Anticoagulants Adulterant’s Identification”, was presented as oral presentation at NEAFS (Northeastern Association of Forensic Scientists) 2020 Virtual Annual Meeting, October 14 – 17, 2020.

A presentation entitled “Screening Anticoagulant Adulterants in Seized Material”, was presented as oral presentation at MAAFS (Mid-Atlantic Association of Forensic Scientists) 2021 Annual Meeting, September 21 – 24, 2021.

A presentation entitled “Development and Validation for Identifying Anticoagulant in Seized Material by High Resolution Orbitrap Mass Spectrometry”, was presented as poster presentation at ASMS (American Society of Mass Spectrometry) 2021 Annual Meeting, October 31 – November 4, 2021.

A presentation entitled “Quantitative Analysis of Anticoagulants in Human Blood by Triple Quadrupole Mass Spectrometry”, was presented as oral presentation at MAAFS (Mid-Atlantic Association of Forensic Scientists) 2022 Annual Meeting, May 10 – 13, 2022.

A presentation entitled “Quantitative Analysis of Anticoagulants in Human Blood by UPLC coupled with Triple Quadrupole Mass Spectrometry”, was presented as poster presentation at ASMS (American Society of Mass Spectrometry) 2022 Annual Meeting, June 5 – 9, 2022.

A presentation entitled “Detection of the Superwarfarin Drugs and Synthetic Cannabinoids in Human Biological Samples”, will be presented as an oral presentation at the VIII NPS Conference 2022 Annual Meeting, October 24 – 26, 2022.

A presentation entitled “Quantitative Analysis of Anticoagulant in Human Blood by UPLC coupling with Triple Quadrupole Mass Spectrometry”, will be presented as poster presentation at SOFT (Society of Forensic Toxicologists) 2022 Annual Meeting, October 30 – November 4, 2022.

Dissemination Activities

Results related to this research have been disseminated at various professional meetings nationally and internationally, which included: the Northeastern Association of Forensic Scientists – NEAFS 2020 Virtual Annual Meeting, Mid-Atlantic Association of Forensic Scientists – MAAFS 2021 Annual Meeting and MAAFS 2022 Annual Meeting, as well as the American Society for Mass Spectrometry – ASMS 2021 Annual Meeting and ASMS 2022 Annual Meeting. Results will also be disseminated at the 2022 VIII NPS Conference and the 2022 Society of Forensic Toxicologists Annual Meeting.

A Superwarfarin Toolkit, which contains all of the extraction protocols, analytical methods and instrumental parameters for both seized drug analysis and toxicology workflows developed as

part of this project was developed and will be publicly accessible on our website (www.cfsre.org) as well as distributed via our extensive email distribution list.

Finally, manuscripts related to the evaluation of current methods for seized drug testing and the development of new methods for the analysis of anticoagulants have been drafted and will be submitted for publication in peer reviewed literature.

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Appendix A

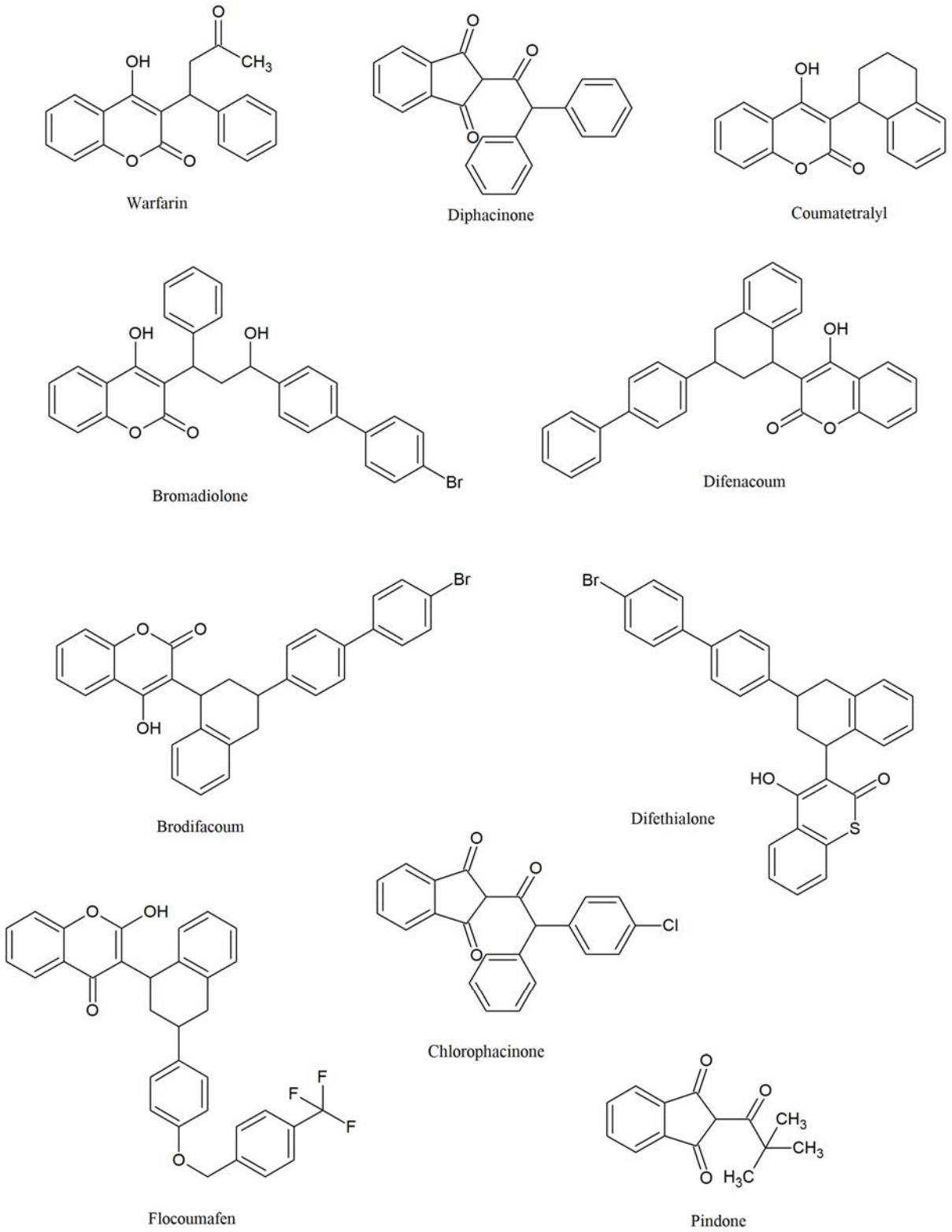

Figure 1. Chemical structure of the target anticoagulant adulterants.

This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.