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Detection of anticoagulant rodenticides by direct analysis in real time time-of-flight mass spectrometry: novel screening techniques and rapid semi-quantitative determination

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Environmental monitoring of rodenticide contamination is often required to address public concern of off-target poisoning, which threatens animal populations and disrupts related ecology. This study aimed to address the shortcomings of current analytical methods for testing water-soluble anticoagulant rodenticide by application of direct analysis in real time in tandem with time-of-flight (DART-ToF) mass spectrometry (MS), an alternative analytical technique offering rapidity, low cost, and robustness. Both positive and negative ion modes were investigated with the aim of establishing a quick screening and semi-quantitation workflow. Screening was conducted by development of a custom DART-ToF MS library with selected rodenticide spectra, and data was analyzed using the National Institute of Standards and Technology/National Institute of Justice DART-MS Data Interpretation Tool (NIST/NIJ DART-MS DIT). The developed methodology readily identified five rodenticides from complex mixtures at 1 mg L^{-1} and one rodenticide at 10 mg L^{-1}. Semi-quantitation was conducted through the internal standard method and negative mode ionization, with linear relationships from $R^2 = 0.98$ to 0.99. While further optimization with alternative internal standards may be considered, this study revealed the analytical potential in DART-ToF for rapid identification and quantitation of many environmental contaminants.

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1. Introduction

Rodenticides are used to address sanitary concerns resulting from mice or rat infestations.[1] However, the commercial use of rodenticides can lead to widespread transfer to unintended environments, causing debilitating effects on humans, wildlife, and natural resources. Anticoagulant rodenticides (ARs) inhibit organismal function through disruption of the vitamin K cycle and diminishing of blood clotting factors, potentially resulting in excessive bleeding.[2] The demand for more potent rodenticides led to the emergence of two distinct classes: first-generation anticoagulant rodenticides (FGARs) and second-generation anticoagulant rodenticides (SGARs). FGARs need multiple feedings to eliminate rodents, while SGARs are more potent and persistent, only requiring one feeding to take effect, but remaining in the tissue for an extended period of time.[3,4] Cases of AR poisoning among humans typically resulted from accidental ingestion[5–8] or drugs of abuse adulteration[9,10] with SGARs, but the consequences of rodenticides are more frequently observed among food chain recipients such as predators of rodents, which is a major environmental concern. Scientists in England, Scotland, Poland, the United States, and Canada have tracked the prevalence of SGARs in birds of prey.[11–15] Moreover, the injection of rabbits with pindone and turtles with warfarin elucidated the persistence of FGARs in tissues of non-rodent animals, which carries major implications for multiple avenues in which rodenticides may endanger wildlife.[16,17] The World Health Organization (WHO) recognized the risk of primary and secondary poisoning of rodent baits,[18] which was further supported in a 2018 review analyzing

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worldwide cases of non-targeted rodenticide poisoning.[3] While ARs can linger in the environment *via* secondary means such as birds, the consequence of exposure is exacerbated by release of rodenticides into surface and ground waters, potentially creating significant damage to the natural flora and fauna. Primus and Regnery previously discussed the prevalence of rodenticide contamination in stormwater, wastewater, and groundwater in Australia, the United Kingdom and Germany.[19,20] Case studies determined rodenticide leakage was associated with elevated levels of rodenticides in water, and traces of rodenticide in tissues of aquatic species. Thus, qualitative and quantitative analysis of rodenticides in aqueous matrices is imperative to support regulating commercial biocides and conservation efforts.

Ever since the range of its off-target effects being recognized, scientists have been working on rodenticide isolation, detection, and quantitation in environmental samples.[21,22] Various chromatographic and spectroscopic methods were developed and applied to rodenticides in biological matrices of afflicted animals and potentially affected water and soils. High-performance liquid chromatography (HPLC) based methods were commonly used in the analysis of rodenticides.[23–38] This was historically preferred over gas chromatography (GC), which performed comparatively poorer due to suspected degradation of ARs at high temperatures and the necessity for derivatization to improve the lower limits of detection.[39] Historically, HPLC with Diode Array Detection (DAD) or Ultraviolet (UV) detection[26,31,38] and fluorescence detection[22,24,26,31,38] was primarily used to determine the presence of rodenticides. The advent of liquid chromatography-coupled mass spectrometry or tandem mass spectrometry (LC-MS or LC-MS/MS) significantly has improved specificity and sensitivity of rodenticide characterization because of the information based on targeted compounds' molecular weight rather than their unique spectral properties.[12,27–30,32–37] Researchers in veterinary, forensic, and environmental sciences applied analytical methods on biological matrices such as blood, urine, feces, and tissue to accurately compare potential rodenticide poisoning in humans and animals.[12,23–26,28,30–36] Other potential sample types such as food and hair can also retain detectable quantities of ARs.[29,37,38] An assessment of surface waters from storm runoffs in Germany detected rodenticides which was traced to sewage leakage into critical aqueous channels. This study was based on using extensive extraction and concentration procedures by dispersive solid-phase extraction (d-SPE) together with LC-MS analysis.[36] However, the current LC and GC based methods require laborious sample preparation, expensive instrumentation, frequent equipment maintenance, and extended processing time. Thus, new approaches such as Direct Analysis in Real-Time (DART) are being explored, which may offer a faster and simpler procedure as a screen tool in comparison to the conventional methods.[40]

DART Mass Spectrometry (DART-MS), first introduced in 2005, offers an ionization method in ambient temperature and has proven to be particularly useful for the analysis of various organic compounds in high-throughput screening circumstances.[40–45] DART-ToF has been widely implemented to expedite forensic testing processes, which is reflective of its potential in environmental monitoring.[46] In DART-ToF, a sample at atmospheric pressure is exposed to the flowing afterglow of a helium glow discharge. The DART gas stream contains helium atoms in long-lived electronically excited states ("metastable atoms"). The internal energy of the metastable helium atoms is sufficient to ionize atmospheric water and oxygen. In positive-ion mode, ionized water clusters $[(H_2O)^n + H]^+$ undergo ion–molecule reactions with analytes with suitable proton affinities to produce protonated molecules $[M + H]^+$. In negative-ion mode, oxygen ions O_2^- can produce deprotonated molecules $[M - H]^-$ from acidic compounds.[10] Rodenticides have been screened with DART-ToF in combination with additional techniques, such as pre-analysis extraction and thermal desorption.[47,48] Other researchers published applications using DART-ToF for a variety of environmental contaminants such as crude oils and petroleum oils, in which classification was accomplished through multivariate statistical analysis on the Mass Mountaineer™ program.[41–45] While a DART MS forensic database is currently available from NIST to identify significant chemical compounds, such as drugs and pharmaceuticals, the database currently lacks mass spectral information for rodenticide identification.[49] Moreover, the NIST library is designed to identify compounds based on the features of a relatively pure sample; In GC/MS, this is accomplished through chromatography and electronic impact ionization. However, DART-ToF poses the challenge of generating spectra in combination of a specific compound together with other matrix components. With environmental samples often containing various compounds from weathering or biological activity, the DART MS forensic database appears ill-suited for complex matrices.

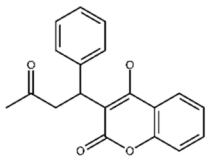
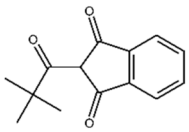
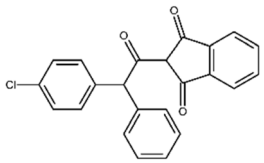
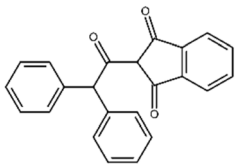
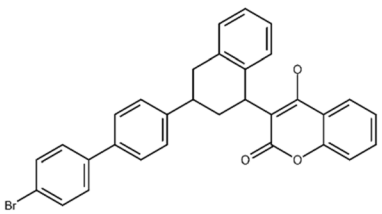
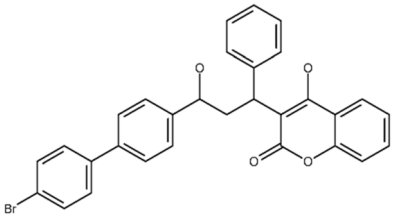
In this study, we develop the first DART mass spectral library of rodenticides, formatted for use with the National Institute for Standards and Technology/National Institute of Justice DART-MS Data Interpretation Tool (NIST/NIJ DART-MS DIT),[50,52] commonly referred to as the DIT.[51] This method of data interpretation aims to expand the practice of DART-ToF rodenticide analysis from previously established LC/MS methods[47,48] to a faster and much simpler alternative procedure. The algorithm underpinning the DIT—the inverted library search algorithm (ILSA)—is designed to identify unique components of mixtures from mass spectra collected from in-source Collision Induced Dissociation (is-CID) DART-MS.[52,53] Having demonstrated success in screening drugs of abuse in forensic and public health sectors,[54–56] the NIST formatted mass spectral library can also provide environmental scientists with a fast, reliable, and accessible resource for monitoring rodenticide levels in samples of interest. Extended with potential "false positive" organic compounds with ion responses in a similar mass range, the DIT should be able to distinguish rodenticides from non-rodenticides with potential of promptly screening samples with distinction from interfering compounds. To our knowledge, these approaches have not been attempted in the past and may lead to a powerful tool for environmental trace organic labs. Additionally, the openness of the DIT source code and library elevates the potential of having all the DART-ToF users able to

contribute spectral information to build a spectral library accessible for all users.

While the prospect of using DART-ToF and the DIT in conjunction with each other may further enhance the qualitative analysis, quantitative analysis of rodenticides using DART-ToF has yet to be fully explored. The first quantitative application of DART was a toxicology study using DART to determine gamma-hydroxybutyrate (GHB) in urine.[56] Others have demonstrated the quantitative capabilities of DART-ToF analysis using an internal standard method with improved precision.[57,58] The deuterated organic compounds decanoic-d_3 acid and *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3, were studied as less expensive and readily available internal standard materials in the analysis of naphthenic acids by LC/QToF.[59] These compounds were selected based on their general availability and volatility to the target compound(s). It is recognized that these potential internal standard compounds have different ionization efficiencies to deuterated rodenticide materials, but the latter are cost prohibitive. To optimize signal intensity, both positive and negative ion mode were evaluated; negative mode on DART-ToF is less explored in prior rodenticide studies using the same analytical technique but may offer much promise given historically successful detections of ARs using negative ion mode on LC/MS analysis. The efficacy of this method was measured by rodenticide deprotonated molecule peak height-to-internal standard peak height in ratio, herein referred to as the IS response ratio.

The objectives of this study were to (1) investigate whether six anticoagulant rodenticides in water could be monitored using DART-ToF MS in both positive and negative ion mode, (2)

Table 1 Rodenticide types, classes, molecular weight (g mol^{-1}) and structure

Rodenticide	Class	Molecular weight (g mol^{-1})	Structure*
Warfarin	SGAR	308.328	
Pindone	FGAR	230.094	
Chlorophacinone	SGAR	374.820	
Diphacinone	SGAR	340.371	
Brodifacoum	FGAR	523.417	
Bromadiolone	FGAR	527.405	

create a mass spectral library which can be used with the DIT to identify rodenticides from simulated environmental water samples, and (3) develop a semi-quantitative method to determine the concentration of rodenticides in surface marine waters.

2. Experimental

2.1. Materials and methods

Six rodenticides listed in Table 1 were characterized in this work: Warfarin and brodifacoum were purchased from Sigma-Aldrich (Oakville, ON, Canada), while pindone, chlorophacinone, diphacinone, and bromadiolone were sourced from ChemService (Chester, PA, USA). In addition, one herbicide and four fungicides were included in this study: boscalid, picoxystrobin, quinoxyfen, and carbetamide were all purchased from Fluka (Morris Plains, NJ, USA) and metsulfuron-methyl was obtained from Sigma-Aldrich (Oakville, ON, Canada). All compounds were dissolved in acetonitrile (SupelCo, Bellefonte, PA, USA) to provide stock solutions at 1000 mg L^{-1} with refrigerated storage at 5–9 °C. Filtered seawater was obtained *via* an underground pump located at the Pacific and Environmental Science Center (PESC) and sourced from the Burrard Inlet (British Columbia, Canada).

Table 2 DART-ToF parameters prior to semi-quantitation method

Parameter	Positive mode	Negative mode
Heater temperature (°C)	350	350
Orifice temperature (°C)	120	150
Detector voltage (V)	2300	2400
Orifice 1 voltage (V)	20	−20
Orifice 2 voltage (V)	+5	−5
Ion guide voltage (V)	500	500
Sampling interval (ns)	0.25	0.25
Recording interval (s)	1	1

2.2. Instrumentation

Rodenticides were analyzed with the AccuTOF™-DART® (JEOL USA, Inc., Peabody, MA, USA) in combination with a DART-SVP ion source (IonSense, Saugus, MA, USA) using a continuous stream of helium gas (Linde, Delta, BC, Canada). This instrument was applied in conjunction with msAxel@LP® data processing software (ver. 1.0.5.2) to control parameters, collect spectra, and process data. Details of the instrument set up conditions are provided in Table S1. Calibration of the instrument was conducted using 0.5% (w/v) Polyethylene Glycol (PEG) 600 (Tokyo Chemical Industry, Tokyo, Japan) in methanol (Sigma-Aldrich, Oakville, ON, Canada), where the corrected $1 - R$ value would be kept between 1×10^{-13} and 9.99×10^{-12}.

Samples were measured by dipping a glass capillary tube (Kimble, Vineland, NJ, USA) in a solution of the compound of interest, removing, and placing the capillary end in the 2 cm gap between the DART gas stream and the MS instrument. The sample was held in this position for approximately 10 s while the msAxel@LP software depicted a rising spectral mass signal. The orifice and DART were frequently cleaned with methanol (SupelCo, Bellefonte, PA, USA) to avoid contamination or blockages.

2.3. Data processing

Rodenticides were used to establish a DART-ToF mass spectral library to work with the DIT. The mass spectral library efficacy was tested using a variety of unknowns and their mixtures. The procedure was first assessed by identification of rodenticide in a sample by reference to the in-house library on the NIST/NIJ DIT, and followed by semi-quantitation by reference to an IS response ratio standard curve for designated compounds. The semi-quantitation process was developed by measuring rodenticide-to-internal standard responses across a concentration gradient, generating linear regression curves based on these responses, and testing its interpolation efficacy with various known and unknown standards.

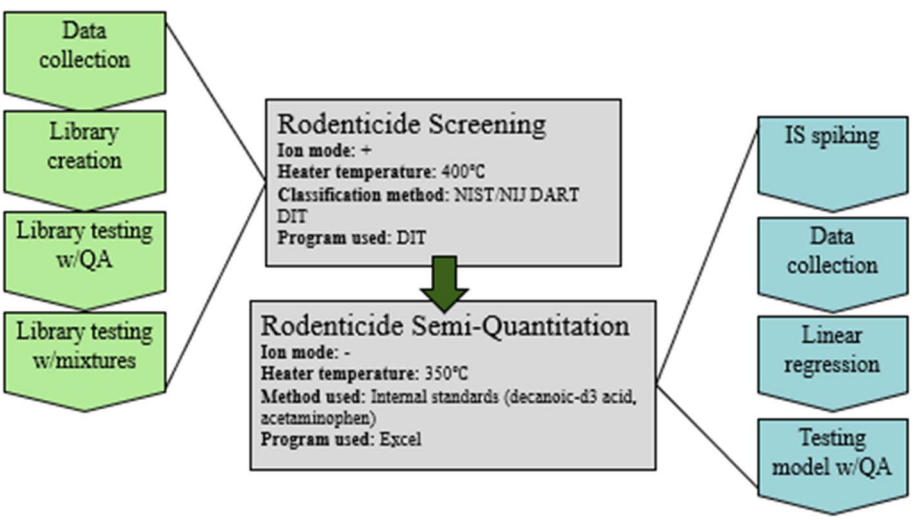


Fig. 1 General workflow of rodenticide analysis (grey) and development methodology (green and blue).

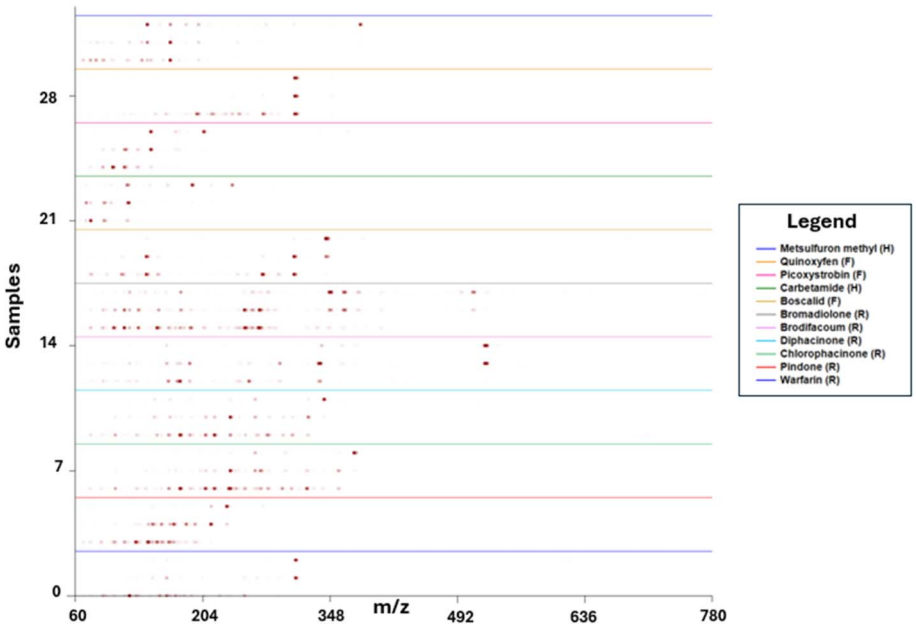


Fig. 2 Heat map of rodenticide (R), fungicide (F) and herbicide (H) reference standards (1000 mg L^{-1}, with the exception of brodifacoum at 50 mg L^{-1}) at low (30 V), medium (60 V) and high (90 V) voltages, from top to bottom respectively. Heat map was generated by Mass Mountaineer™ software (Ver. 7.1.25).

2.4. NIST-formatted library

Using the AccuTOF™-DART® in positive ion mode, the parameters were set to comply with NIST spectral library requirements:[48] a heater temperature of 400 °C and an orifice temperature of 120 °C were employed. Detector voltage was set to 2300 V; Orifice 2 voltage was set to −5 V and ion guide voltage set to 800 V. The sampling interval was kept at 0.25 ns and recording interval at 0.4 s. Parameter switching was implemented to change the orifice 1 and ring lens voltage. Orifice 1 voltage switched between +20 V, +30 V, +60 V, and +90 V. For +20 V, +30 V and +60 V the ring lens voltage was fixed at 5 V, while at an orifice 1 and voltage of +90 V, the ring lens voltage was at 10 V. Caffeine d9 was used as performance verification standard, which was prepared with 10 mg L^{-1} caffeine d9 (CDN Isotopes, Pointe-Claire, QC, Canada) in acetonitrile and run by measuring the peak intensity of five replicates and ensuring their peak heights were above 10^5 in arbitrary units.

Rodenticide, herbicide, and fungicide mass spectra were collected to build an in-house DART-ToF spectral library. To generate valid mass spectra complying with NIST parameters, triplicate sampling of rodenticides or single sampling fungicides and herbicides with a concentration of 1000 mg L^{-1} were measured using the Accu-ToF 4G at multiple voltages, with capillary tube blanks and PEG 600 reference material confirming background and mass calibration between different compounds. Final data extraction was conducted in drift compensation mode, adjusting mass-to-charge ratios (m/z) such that all data was calibrated in accordance with PEG 600 calibration data files in positive and negative mode. The spectra of measured rodenticides were first subtracted by capillary tube background, then saved as centroided text files, and sorted into a library for assembly in the DIT. Using a custom R script (available through correspondence with corresponding authors), the library was generated in a traditional format as an RDS file before being uploaded to the DIT's working directory of libraries.

The collated library was assessed by analyzing the internal quality assurance (QA) and quality control (QC) samples. For QA tests, an alternate analyst prepared single rodenticide solutions as blind study samples. For QC tests, two known rodenticides were combined for analysis. The unknown QA samples were prepared as follows: single rodenticide standards were diluted with ultra-high purity (UHP) water or seawater from their 1000 mg L^{-1} stock solution to provide concentrations ranging from 1–100 mg L^{-1} for analysis. QC mixtures were prepared in ratios of 1 : 1, 1 : 3, and 3 : 1 of randomized combinations from 100 mg L^{-1} rodenticide stock mixtures. These samples were measured in triplicates and data was extracted as centroided text files. Once collected and extracted, the mass spectra (+30 V, +60 V, +90 V) of QA and QC samples were uploaded to the DIT. With the in-house rodenticide library selected and based on the characteristic fragmentation ions, the QC and QA were determined with the DIT software.

2.5. Rodenticide semi-quantitation by internal standard response ratio

Two sets of parameters were tested for semi-quantitation of rodenticides with parameters listed in Table 2.

Internal standards decanoic-d3 acid and *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3 (CDN isotopes, Pointe-Claire, QC, Canada) were prepared separately in acetonitrile, each at a concentration of 10 000 mg L^{-1}. Serial dilution of each rodenticide stock solution (100 mg L^{-1}) produced working standard solutions with concentrations of 3.125, 6.25, 12.5, 25,

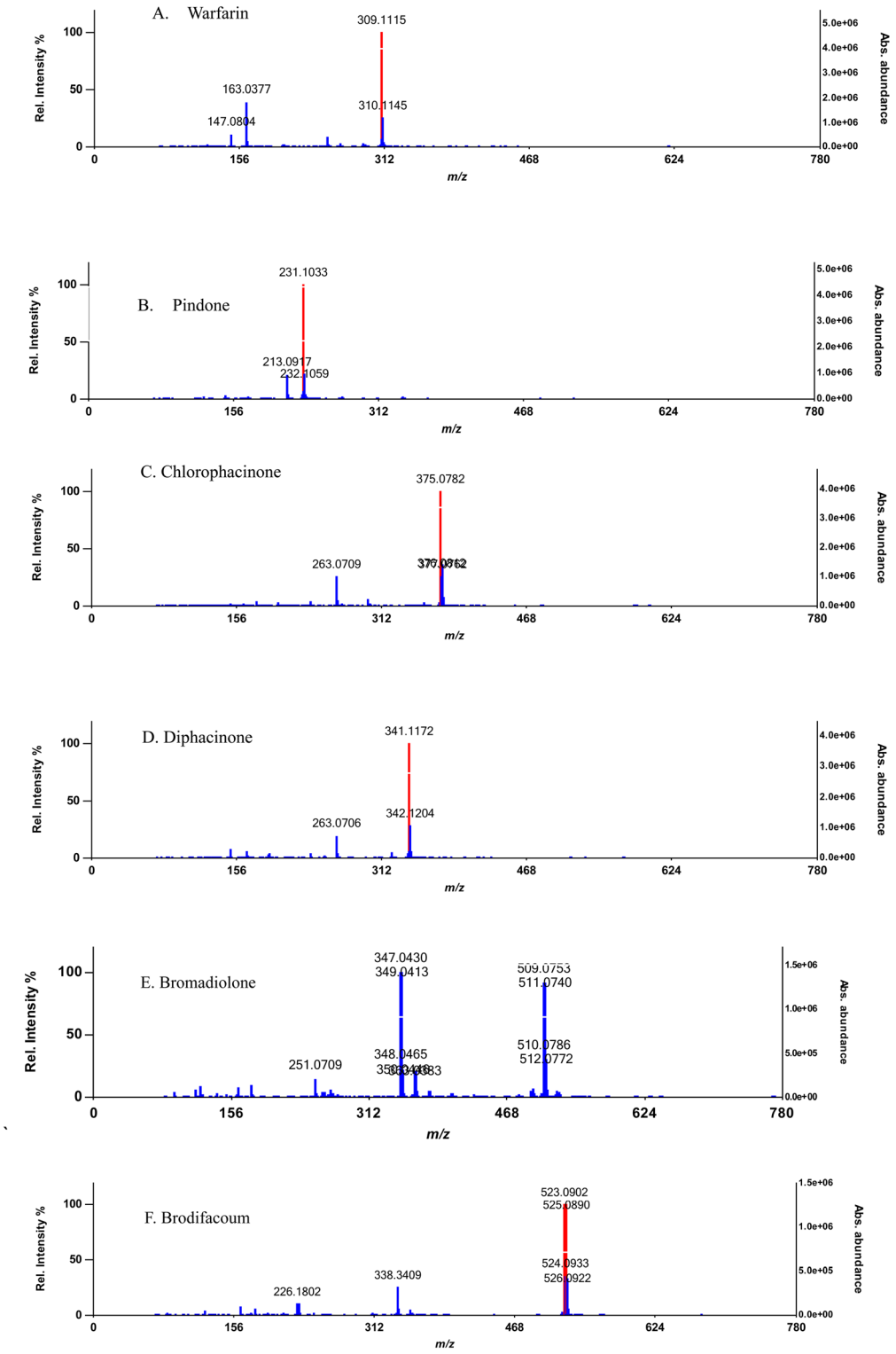


Fig. 3 Mass spectra of rodenticides at 1000 mg L^{-1} (A) warfarin, (B) pindone, (C) chlorophacinone, (D) diphacinone, (E) bromadiolone) and 50 mg L^{-1} (F) brodifacoum) and collected by DART-ToF MS in positive ion mode at +30 V with the glass capillary background subtracted. Data was visualized from a centroided text file using Mass Mountaineer™, where relative intensities were mapped against *m*/*z*. The red peak indicates the *m*/*z* used for protonated molecule comparisons.

50, and 100 mg L^{-1}. Using positive displacement pipettes, each rodenticide working solution was then spiked with the same final concentration of internal standards ranging from 20 to 100 mg L^{-1}. The following combinations yielded a linear regression model with $R^2 \geq 0.98$: Warfarin at 20 mg L^{-1} of *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3, bromadiolone at 100

mg L^{-1} of deuterated *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3, pindone at 20 mg L^{-1} of decanoic-d3 acid, and chlorophacinone at 20 mg L^{-1} of decanoic-d3 acid.

Following PEG 600 calibration, 6 replicates of each internal standard-spiked rodenticide standard was analyzed on the Accu-ToF 4G, with each set of replicates separated by a PEG 600 mass calibration check. Data was extracted as previously described. IS response ratio was calculated by dividing the major ion peak intensity of the rodenticide by that of the internal standard. The nominal rodenticide concentration *vs.* average IS response ratio was plotted with standard deviation.

To evaluate the accuracy of the internal standard method, rodenticides of unknown concentrations were spiked with their designated internal standard and measured on the Accu-ToF. An average of 6 replicates' IS response ratios were interpolated to determine an approximate semi-quantitative concentration from two methods: Method 1 derived a final concentration from the averages of six IS response ratios, while method 2 derived a final concentration from the averages of 6 rodenticide peak heights and 6 internal standard peak heights. Statistical analysis was conducted using a two-tailed *T* test where $\alpha = 0.002$.

Table 3 Mass comparison of calculated value to DART-ToF measured protonated molecule $[M + H]^+$. Average, standard deviation and relative standard deviation are calculated from triplicate mass values of each compound. Fungicides and herbicides were not measured in triplicates, thus columns for standard deviation and relative standard deviation were not included. Bolded cells indicate that average mass value is within 0.005 Da of the true value

	$[M + H]^+$ (*m*/*z* or Da)			
Compound	True mass	Average	Standard deviation	Relative standard deviation
Warfarin	309.1126	309.1126	0.00108077889	0.000349639230%
Pindone	231.1021	231.1032	0.000937535244	0.000405678128%
Chlorophacinone	375.0788	375.0782	0.0000745944591	0.0000198877087%
Diphacinone	341.1178	341.1172	0.000208893115	0.0000612379366%
Bromadiolone	527.0858	347.0425[a]	0.000669630122	0.000192953329%
Brodifacoum	523.0909	523.0917	0.001312082441	0.000250832208%
Boscalid	343.0327	343.0380		
Picoxystrobin	368.1031	368.1091		
Quinoxyfen	308.0039	308.0020		
Metsulfuron-methyl	382.0743	382.0777		
Carbetamide	237.1231	237.1217		

[a] Indicates the highest intensity peak *m*/*z* of bromadiolone. While it is not indicative of the protonated molecule, this value is included for precision determination.

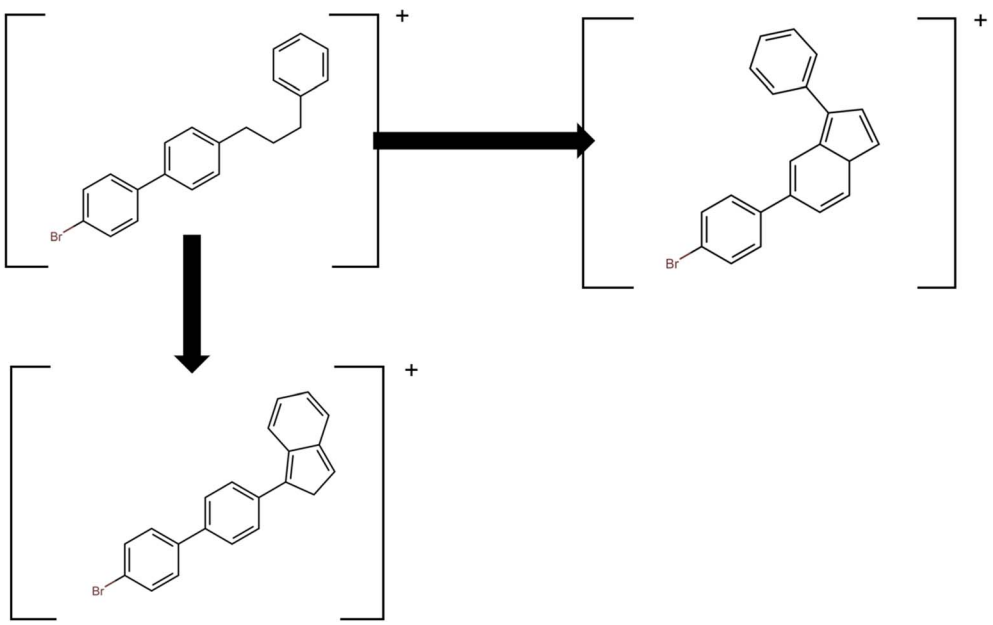


Fig. 4 Suspected bromadiolone fragments. The existing fragment (top left) is suspected to cyclize in two proposed ways (right and bottom) while retaining a detectable positive ion charge.

3. Results and discussion

3.1. Rodenticide screening by NIST DIT

The current study was conducted to develop a practical methodology for the detection of rodenticides in environment samples using DART/ToF. Therefore, the method development followed a 2-step approach, *i.e.*, qualitative with the use of in-house spectral library and semi-quantitative with the use of internal standard method under negative mode (Fig. 1).

To establish a rodenticide specific spectral library, the standards were first run with multiple cone voltages. The purpose of acquiring rodenticide mass spectra at +30 V, +60 V and +90 V was to elucidate the precursor and various product ions in full clarity (Fig. 2). The precursor ion identity was to reference the molecular weight of the protonated molecule in the +30 V spectrum as shown in Fig. 3, with fragment ions increasing in intensity with increased applied voltages. Table 3 demonstrated that clear and reproducible reference spectra were generated using the DART-ToF, with 8 of 11 compound mass-to-charge ratios within 0.005 Da of the true value. Note that a limited supply of the brodifacoum rodenticide made analysis results only available at 50 mg L^{-1}, although future standardization at 1000 mg L^{-1} is recommended. The lower concentration of this rodenticide did not appear to affect its final assessment during method quality assessment in the current study. Bromadiolone had an unexpected ion peak at 347.0 *m*/*z*, which outperformed its protonated molecule at *m*/*z* 527.1 *m*/*z* in intensity (Table 3, and Fig. 3e). The peak at *m*/*z*

Table 4 Computational interpretation of QA rodenticides using the DIT

Rodenticide		Warfarin	Warfarin	Warfarin	Pindone	Pindone	Chlorophacinone	Chlorophacinone
Concentration (mg L^{-1})		50	5	1	100	1	50	1
Replicate								
1	Top target[a] Δ *m*/*z*	Warfarin −0.0007	Warfarin 0.0003	Warfarin 0.0004	Pindone −0.0005	Pindone 0.0003	Chlorophacinone −0.001	Chlorophacinone −0.0012
2		Warfarin −0.001	Warfarin 0.0004	Warfarin 0.0006	Pindone −0.0006	Pindone 0.0005	Chlorophacinone −0.001	Chlorophacinone −0.0007
3		Warfarin −0.0008	Warfarin 0.0004	Warfarin 0.0001	Pindone −0.001	Pindone 0.0007	Chlorophacinone −0.0016	Chlorophacinone −0.0011

Rodenticide		Diphacinone	Diphacinone	Diphacinone	Bromadiolone	Bromadiolone	Brodifacoum
Concentration (mg L^{-1})		100	20	1	50	1	1
Replicate							
1	Top target Δ *m*/*z*	Diphacinone −0.0009	Diphacinone −0.001	Carbetamide 0.1000	Bromadiolone 0.0000	Carbetamide −0.0312	Brodifacoum 0.0000
2		Diphacinone −0.0014	Diphacinone −0.0015	Diphacinone −0.0001	Bromadiolone 0.0000	Carbetamide −0.0315	No target N/A
3		Diphacinone −0.0012	Diphacinone −0.0002	Diphacinone −0.013	Metsulfuron-methyl 0.0131	Carbetamide 0.1	Brodifacoum −0.0008

[a] "Target" refers to the identified compound based on the highest intensity peak. The resulting *m*/*z* error for the most prominent identifying peak is included below the target name. Bolded font indicates a false positive herbicide or fungicide was identified, or it indicates no target was found. Minimum target threshold = 1% and maximum *m*/*z* tolerance = 0.1 Da.

Table 5 Computational interpretation of QA samples in seawater using the DIT

Rodenticide		Bromadiolone	Brodifacoum	Chlorophacinone	Diphacinone	Warfarin	Pindone	Bromadiolone
Concentration (mg L^{-1})		1	5	5	10	25	5	10
Replicate								
1	Top target[a] Δ *m*/*z*	Metsulfuron-methyl 0.017	Brodifacoum −0.0032	Chlorophacinone −0.0002	Diphacinone 0.0009	Warfarin −0.0006	Pindone 0.0021	Metsulfuron-methyl −0.0459
2		Metsulfuron-methyl 0.0178	Brodifacoum −0.0017	Chlorophacinone −0.0003	Diphacinone 0.0016	Warfarin −0.0009	Pindone 0.0019	Bromadiolone −0.0145
3		Metsulfuron-methyl 0.0162	Brodifacoum −0.0017	Chlorophacinone −0.0004	Diphacinone 0.0015	Warfarin 0.0004	Pindone 0.0018	Bromadiolone −0.0148

[a] "Target" refers to the identified compound based on the highest intensity peak. The resulting *m*/*z* error for the most prominent identifying peak is included below the target name. Bolded font indicates a false positive herbicide or fungicide was identified. Minimum targeting threshold = 1% and maximum *m*/*z* tolerance = 0.05 Da.

Table 6 Computational interpretation of QC mixtures using the DIT

Rodenticide 1		Chlorophacinone			Warfarin			Warfarin		
Concentration (mg L^{-1})		50	25	75	50	25	75	50	25	75
Rodenticide 2		Pindone			Diphacinone				Pindone	
Concentration (mg L^{-1})		50	75	25	50	75	25	50	75	25
Replicate										
1	Target[a] 1	Pindone	Pindone	Pindone	Warfarin	Warfarin	Warfarin	Warfarin	Pindone	Warfarin
	Target 2	Chlorophacinone	Chlorophacinone	Chlorophacinone	Diphacinone	Diphacinone	Diphacinone	Pindone	Warfarin	Pindone
2		Pindone	Pindone	Pindone	Warfarin	Warfarin	Warfarin	Pindone	Pindone	Warfarin
		Chlorophacinone	Chlorophacinone	Chlorophacinone	Diphacinone	Diphacinone	Diphacinone	Warfarin	Warfarin	Pindone
3		Pindone	Pindone	Chlorophacinone	Warfarin	Warfarin	Warfarin	Pindone	Pindone	Warfarin
		Chlorophacinone	Chlorophacinone	Pindone	Diphacinone	Diphacinone	Diphacinone	Warfarin	Warfarin	Pindone

Rodenticide 1		Bromadiolone			Chlorophacinone			Diphacinone		
Concentration (mg L^{-1})		50	25	75	50	25	75	50	25	75
Rodenticide 2			Pindone			Brodifacoum			Brodifacoum	
Concentration (mg L^{-1})		50	75	25	50	75	25	50	75	25
Replicate										
1	Target 1	Pindone	Pindone	Pindone	Chlorophacinone	Brodifacoum	Chlorophacinone	Diphacinone	Diphacinone	Diphacinone
	Target 2	N/A	N/A	N/A	Brodifacoum	Chlorophacinone	Brodifacoum	N/A	N/A	N/A
2		Pindone	Pindone	Pindone	Chlorophacinone	Brodifacoum	Chlorophacinone	Diphacinone	Diphacinone	Diphacinone
		N/A	N/A	N/A	Brodifacoum	Chlorophacinone	Brodifacoum	N/A	N/A	N/A
3		Pindone	Pindone	Pindone	Chlorophacinone	Brodifacoum	Chlorophacinone	Diphacinone	Diphacinone	Diphacinone
		N/A	N/A	N/A	Brodifacoum	Chlorophacinone	Brodifacoum	N/A	N/A	N/A

[a] "Target" refers to the compound suggested by the DIT algorithm. "N/A" in bolded font indicates a second rodenticide was not identified. Minimum targeting threshold = 1% and maximum m/z tolerance = 0.1 Da.

347.0 is suspected to be a fragment of the composition $C_{21}H_{16}Br^+$, which has the potential to cyclize (Fig. 4). The peak at m/z 509.1 likely corresponds to $[M + H - H_2O]^+$, where loss of water from the protonated molecule may have occurred. The relatively low intensity of the protonated molecule is unsurprising, as compounds containing hydroxyl substituents often dehydrate in positive-ion DART ionization. Although these measurements were successfully obtained with a DART heater temperature of 350 °C, temperature optimization may be considered for future studies. In cases of analysis by thermal desorption apparatus, lowering the temperature has previously proven successful for bromadiolone.[4] Overall, mass spectral data collected for 1000 mg L^{-1} rodenticide samples comply with NIST parameters and are acceptable for use in a released NIST library.

The developed DIT's ability to identify rodenticides from 1–100 mg L^{-1} in aqueous solution was measured through the following tests: (1) quality assurance (QA) testing of singular blind rodenticide using a collated library of both rodenticides and additional potential false positive materials (boscalid, picoxystrobin, quinoxyfen, carbetamide, and metsulfuron-methyl), and (2) quality control testing of mixtures of the 6 rodenticides. Tables 4–6 summarize the results of the DIT interpretations for the tests. Majority of the samples, 13 out of 14 randomized blind QA rodenticides in UHP water were successfully identified at a targeting threshold of 1% and minimum m/z tolerance of −0.0016 Da. Predictably, brodifacoum and bromadiolone would be recognized at higher m/z tolerances than other compounds given their recorded difficulty in mass spectral recovery at high temperatures.[37] Brodifacoum showed one in three non-target results, most likely due to its low concentration. For diphacinone, two of three replicates showed a positive result. For rodenticide samples in seawater, five of six rodenticides were identified with a maximum m/z tolerance of 0.0021 Da. Bromadiolone was recognized at 10 mg L^{-1} with a maximum m/z tolerance of 0.05 Da, further corroborating the differential ability of the DIT. In general, the identification of rodenticide QA samples was repeatable, with DIT results showing precision and accuracy to their targets, even in environmental water matrices. Completion of this with minimal interference by false positive compounds demonstrated the ability of the DIT to identify rodenticides in complex aqueous solutions. In fact, the DIT is capable of this selectivity due to the compounding determination power permissible with the use of the DART-ToF instrument's voltage switching method. Overall, the DIT's reliability for distinguishing rodenticides from potential interfering compounds despite class similarities is well demonstrated with five of six rodenticides being correctly determined at 1 mg L^{-1}.

While singular rodenticides were well characterized using this developed method, Table 6 indicated that the DIT's interpretation of mixtures was generally accurate with some limitations. While four of the rodenticides were identified throughout various binary mixtures, brodifacoum and bromadiolone again stood out as notably different. At all mixture dilutions (1 : 3, 1 : 1, 3 : 1), brodifacoum could be detected in the presence of chlorophacinone but not when combined with diphacinone

Table 7 Comparison of original and basic DIT library for bromadiolone detection[a]

Rodenticide		Bromadiolone							
Matrix		UHP water		Seawater		UHP with pindone			
Concentration (mg L^{-1})		1	1	1	1	50	25	75	
Library		Original	Basic	Original	Basic	Basic			
Replicate	Top target Δ m/z					Target 2 only			
1		Carbetamide	Bromadiolone	Metsulfuron-methyl	Bromadiolone	Bromadiolone	Bromadiolone	Bromadiolone	Bromadiolone
		−0.0312	0.0008	0.017	0.0013	0.0001	0.0559	0.0014	
2		Carbetamide	Bromadiolone	Metsulfuron-methyl	Bromadiolone	Bromadiolone	No target	Bromadiolone	
		−0.0315	0	0.0178	0.0016	0.0008	N/A	0.0015	
3		Carbetamide	Bromadiolone	Metsulfuron-methyl	Bromadiolone	Bromadiolone	Bromadiolone	Bromadiolone	
		0.1	−0.0004	0.0162	0.0029	0.0009	0.0018	0.0009	

[a] "Target" refers to the compound suggested by the DIT algorithm. Bolded font indicates a false positive herbicide or fungicide was identified. "N/A" in bolded font indicates a second rodenticide was not identified. For basic library entries: minimum targeting threshold = 2% and maximum m/z tolerance = 0.005 Da.

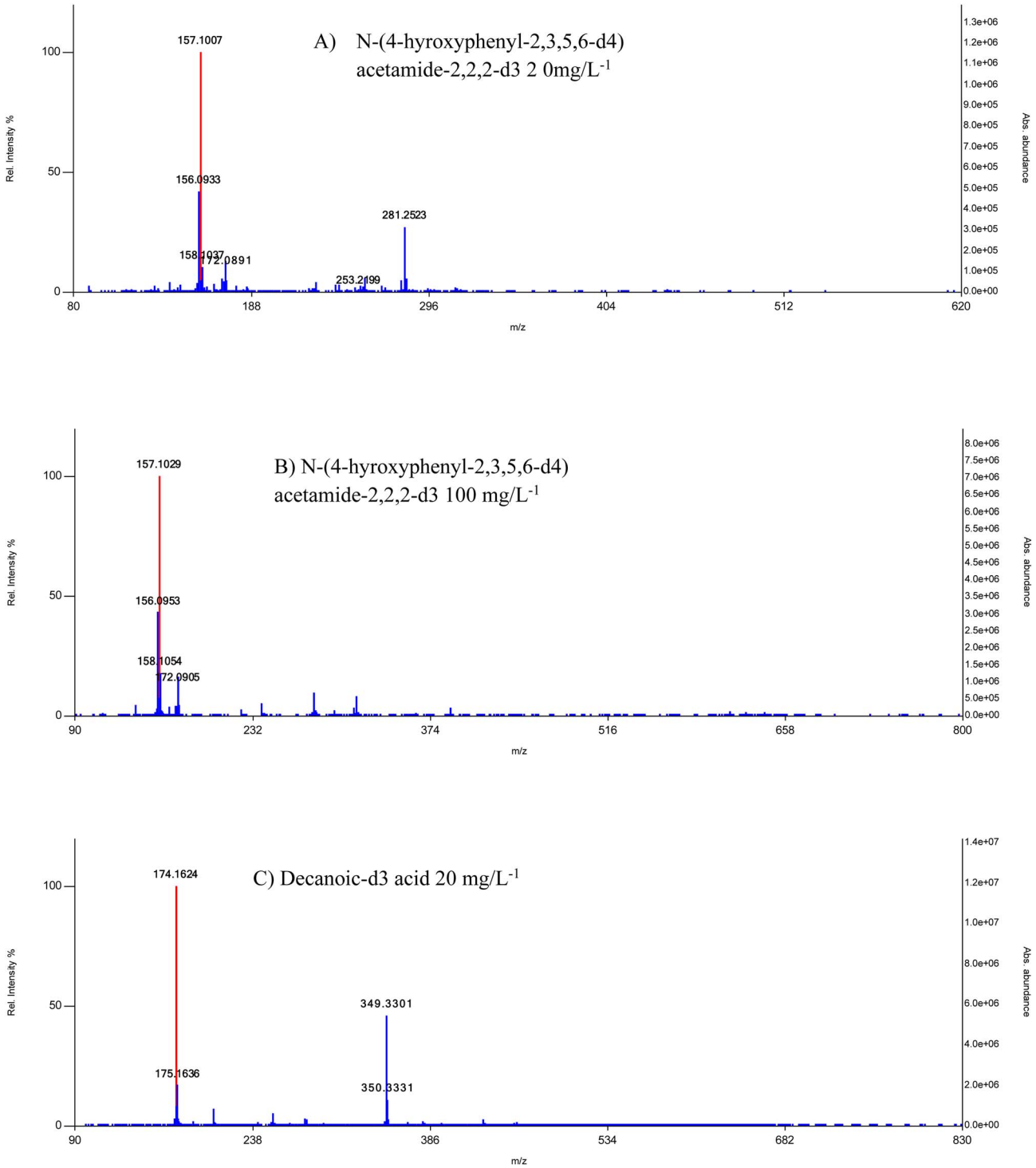


Fig. 5 Mass spectra of (A) 20 mg L^{-1} *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3, (B) 100 mg L^{-1} *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3, and (C) 20 mg L^{-1} decanoic-d3 acid collected by DART-ToF MS in negative ion mode, with water and glass capillary background subtracted. Data was visualized from a centroided text file using Mass Mountaineer™, where relative intensities were mapped against *m*/*z*. The red peak indicates *m*/*z* used for IS response ratio with the rodenticide molecular ion peak.

(Table 6). This again may be attributed to the low concentration due to limited availability as mentioned earlier. It can be concluded that in some circumstances, the detection of low concentrations of this rodenticide may well be interfered with by some, although not all, isobaric compounds that may be present in unknown mixtures.

With respect to bromadiolone's misinterpretation, the likely cause is how the ILSA operates, since it prioritizes identifying

a protonated molecule peak over similarity to library reference spectra. As indicated in Fig. 3, the molecular ion of bromadiolone could not be obtained with DART-ToF conforming to NIST parameters due to its heat sensitivity and observed stability in cyclized fragments. For these unique circumstances, a basic version of the DIT library may be created; unlike the original library which utilizes the SMILES structure of each compound, the basic library requires manual inputting of the target ion (Table 7). As such, a fragment of bromadiolone may be utilized for comparison, which allowed for more effective analysis. The shortfall of this method is that without proper NIST standards in place, this library requires thorough verification to avoid misuse or misidentification of a compound's target ion. Previous more in-depth analytical methods generally employed LC or UPLC MS/MS analysis after extensive sample clean-up and pre-concentration steps and generally reach low ppb or ppt levels. The intent of our current method was to introduce the potential for a rapid screening instrument rather than qualify the method detection limits in various matrices employing extensive clean up procedures before the testing. With the addition of sample clean-up and pre-concentration, there is no reason why similar detection limit levels cannot be met by DART-ToF. As such, the current method could be applied to environmental waters including agricultural run-off, with further potential in application to solid samples such as soils that often become contaminated with bait rodenticides, affecting local wildlife. Noting the potential deficiencies observed in the current study, it was still concluded that DART-ToF analysis with DIT interpretation would outperform traditional lengthier analyses for rodenticide screening by its speed and ability as screening tool for environmental testing labs.

3.2. Rodenticide quantitation by internal standard

Unlike GC/MS or LC/MS, DART/ToF excels in rapid identification of unknown compounds but is not commonly used as a quantitative tool due to the nature of the instrument, *i.e.*, open ionization source, small and unspecific amount of sample being used, and the position and time span of the sample during the ionization process. In other words, the ion signals generated from DART/ToF analysis could vary significantly when running the same sample repeatedly. Therefore, an internal standard method was experimented with to explore the potential of DART/ToF as a semi-quantitative tool, which can assist front-line enforcement officers seeking to examine the level of the pollution in addition to identity of contaminants. It is noted that while there are no specific national guidelines in Canada for the studied rodenticides, this does not mean that the potential for their presence should be ignored. Meanwhile, while most DART/ToF applications are based on positive mode ionization,[41–45] the negative mode ionization in the quantitative area has not been fully explored. Considering rodenticides are commonly analyzed by negative mode LC/MS, it was decided that it would be advantageous to compare the results on both positive and negative mode to determine the suitable approach for semi-quantitative determination of rodenticides in environmental samples with DART/ToF.

All the potential internal standards were run first to generate specific relevant spectral information. Blank DART-ToF mass spectra of internal standards were absent of characteristic masses of the rodenticides studied, minimizing the possibility of false positives in data (Fig. 5). Example rodenticide mass spectra are presented in Fig. 6, and it was noted that

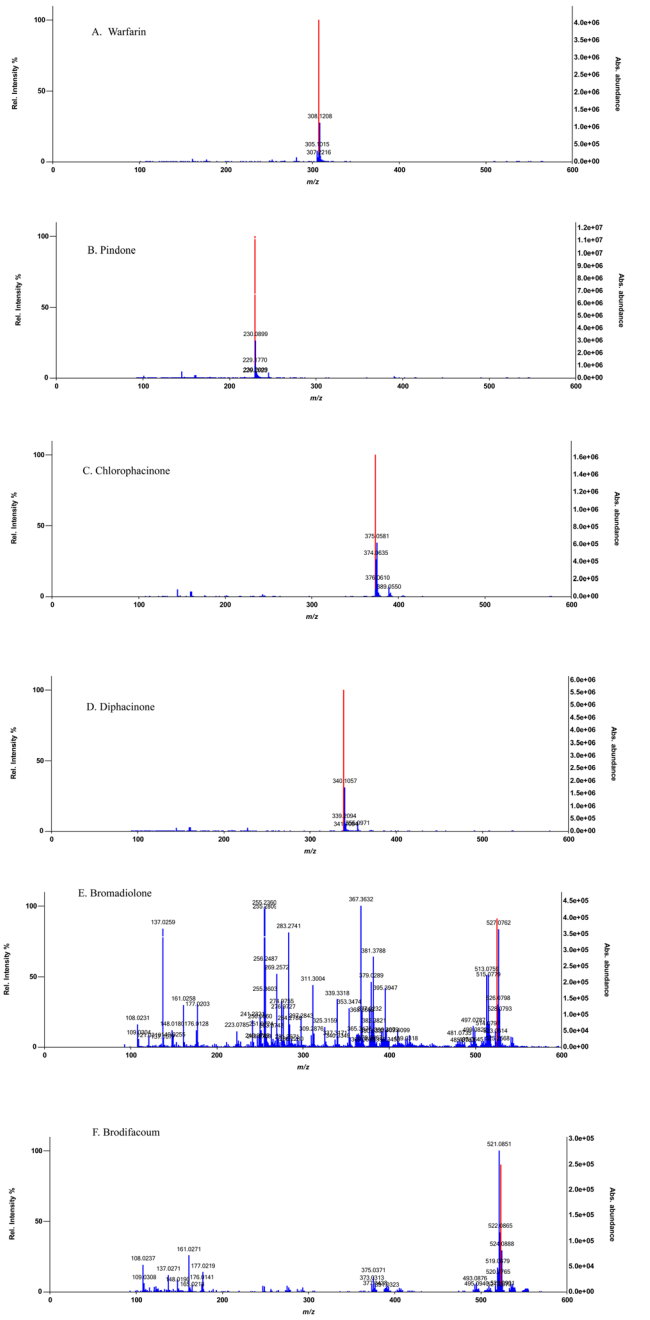


Fig. 6 Mass spectra of rodenticides at 100 mg L^{-1} (A) warfarin, (B) pindone, (C) chlorophacinone, (D) diphacinone, (E) bromadiolone, and 50 mg L^{-1} (F) brodifacoum) and collected by DART-ToF MS in negative ion mode at −20 V with the water and glass capillary background subtracted. Data was visualized from a centroided text file using Mass Mountaineer™, where relative intensities were mapped against *m/z*. The red peak indicates the *m/z* used for IS response ratio with the rodenticide's molecular ion peak.

Table 8 Comparison of base peak intensity between DART-ToF positive and negative ion mode

Rodenticide		Protonated molecule: Positive ion $[M + H]^+$	Deprotonated molecule: Negative ion $[M - H]^+$
Warfarin	*m/z*	309.1125	307.1173
	Peak intensity[a]	**27 335 234**	4 488 163
Pindone		231.1021	229.0885
		8 766 722	**12 473 542**
Chlorophacinone		375.0770	373.0605
		1 476 485	**1 790 409**
Diphacinone		341.1168	339.1028
		4 625 902	**6 151 822**
Bromadiolone		Not found	367.3762
		N/A	**478 292**
Brodifacoum		523.0881	521.0851
		95 210	**304 299**

[a] Peak intensity is measured in arbitraty units. Bolded font indicates the highest intensity between positive and negative ion mode. Spectra of positive ion mode rodenticides may be found in the SI.

bromadiolone, while still exhibiting the molecular ion, showed some potential degradation from storage or fragmentation. Noticeably, negative ion spectra had notably higher signals than positive ion mode for most rodenticides (Table 8, and Fig. 7). Additionally, the lack of visibility of bromadiolone's protonated molecule ion peak made negative mode a stronger contender for quantitative analysis (Table 8). The increased prevalence in the molecular ion at $[M - H]^-$ compared to its lack of counterpart of $[M + H]^+$ in positive ion mode suggests that phenolic groups in hydroxycoumarins display acidic activity more readily, donating protons to form the deprotonated molecule $[M - H]^-$. The protonated molecule of bromadiolone was not detected in positive ion mode, and thus negative ion mode was chosen for semi-quantitation.

As mentioned, that DART/ToF quantitative analysis is better with internal standard run together with the targeted compounds. With repeated optimization, it was determined that internal standard concentration of 20 mg L^{-1} was most suitable for this method development. In fact, this concentration was selected as being suitably within the calibration range of 3.125, 6.25, 12.5, 25, 50, and 100 mg L^{-1} for the rodenticides and would not cause significant competition for ionization. With deuterium ions, the peaks highlighted in red accurately reflect the negative ion of *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3 and decanoic-d3 acid, respectively. In decanoic-d3 acid, additional peaks at *m/z* 349.3 and 250.3 are observed. The peak at *m/z* 349.3 is suspected to be the gas-phase dimer $[2M - H]^+$, which forms from the joining of two carboxyl groups. The peak at *m/z* 250.3 is not clearly identified but its elemental composition is consistent with a gas-phase cluster ion of deprotonated decanoic acid with CO_2 and H_2O. There are additional peaks present, indicating possible fragmentation or contamination. Four rodenticides were selected for this portion of the study and IS response ratios were measured as a dependent variable based on rodenticide concentrations, generating the linear regression curves in Fig. 8. Acceptable internal standard ratio curves with a R^2 of 0.99 were achieved for chlorophacinone, bromadiolone, and warfarin; an R^2 of 0.98 was achieved for pindone. Considering DART/ToF as being mostly a qualitative instrument, this high level of linearity is remarkable. Clearly, for environmental trace organic analysis, the limit of quantitation needs improvement, and much research is needed.

In comparison to the traditional GC/MS or LC/MS, the true advantage of DART/ToF analysis is its rapidity with each run taking only 10 seconds. Therefore, despite a high standard

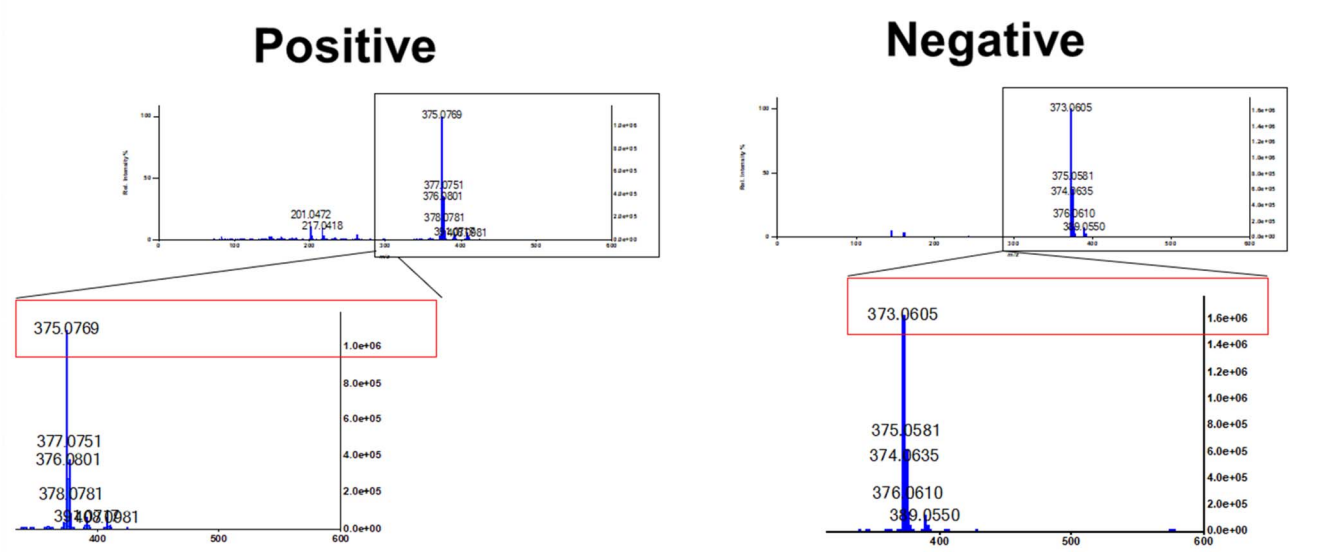


Fig. 7 Sample comparison of positive (left) and negative (right) mode on DART-ToF to analyze 100 mg L^{-1} chlorophacinone. The red box highlights signal intensity of the base peak between both modes.

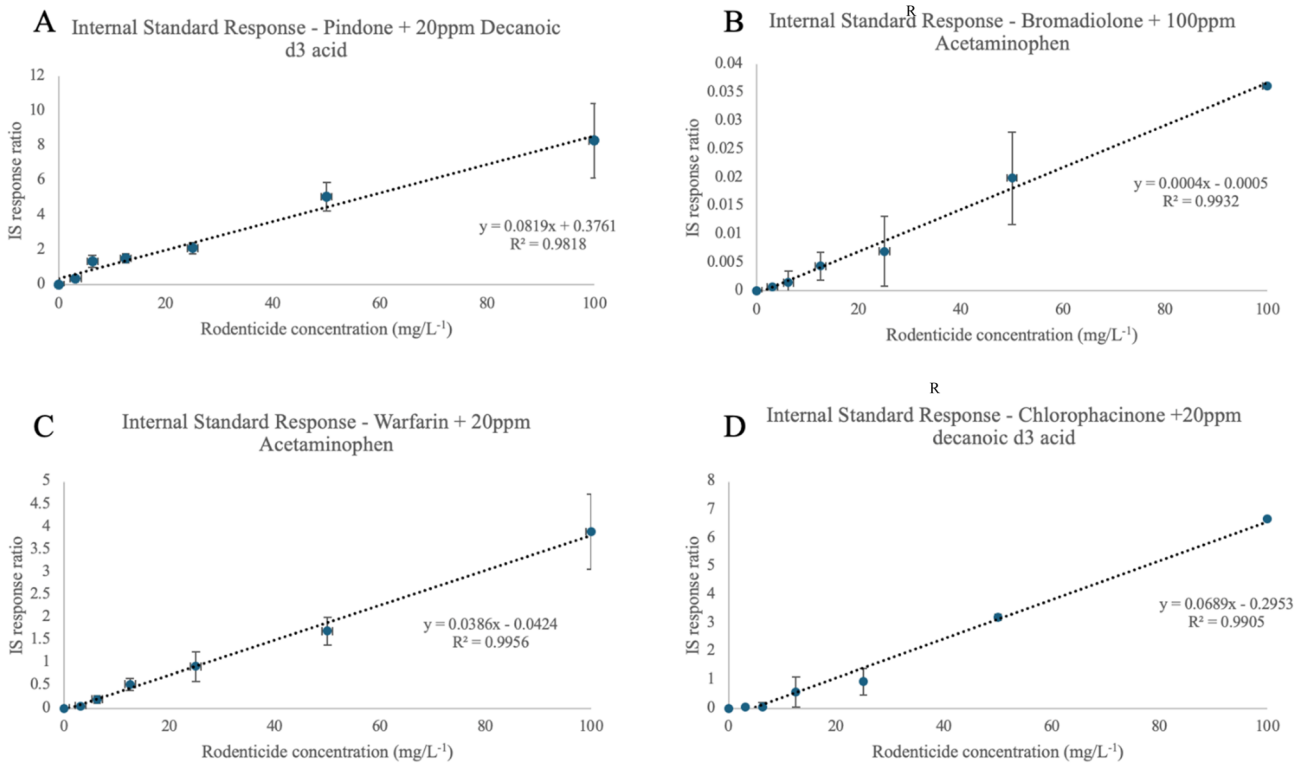


Fig. 8 Linear regression curves describing the relation between rodenticides pindone (A), bromadiolone (B), warfarin (C) and chlorophacinone (D)] and the IS response ratio where $R^2 \geq 0.98$. The internal standard *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3 has been shortened to "Deuterated Acetaminophen". Data points are a collective mean value of 6 replicate IS response ratios per rodenticide concentration. Error bars signify the standard deviation. A line of best fit and R^2 was calculated from Microsoft Excel. The linear relation is displayed on the bottom right of each graph, where y = IS response ratio and x = concentration of rodenticide in mg L^{-1}. Additional figures are available through SI.

deviation was frequently observed between the results of replicates (Fig. 6), the higher number of replicates allowed for an average value achievable in short time, generating statistically reliable signal information for semi-quantitative evaluation. Based on the results from QA sample analysis, it was determined that samples required 6 replicates to be averaged to achieve a more precise measurement. The results for semi-quantitation of blind QA samples are provided in Table 9, demonstrating that 5 of 8 rodenticide solutions of unknown concentration were close to the true value when two internal standards were used. The unexpectedly high concentration of 10 mg L^{-1} bromadiolone was not found to be statistically significant by the two-tailed *T* test, likely due to the substantially larger uncertainty which allowed the estimated range to fall within confidence limits. It should be noted that the two-tailed *T* test is used as a diagnostic method for close estimations only. However, if only one internal standard was used, the results were generally poor and unacceptable, as expected due to the afore-mentioned standard deviation. It was established that this rapid semi-quantitative method analyzes at least six replicates

Table 9 Summary of QA tests involving known rodenticides of unknown concentrations[a]

Rodenticide	True concentration (mg L^{-1})	Method 1 – calculated concentration (mg L^{-1})	Method 1 – test statistic	Method 2 – calculated concentration (mg L^{-1})	Method 2 – test statistic
Warfarin	50	4.02 ± 1.65	−68.11*	3.83 ± 1.34	−84.12*
	10	0.77 ± 0.33	−68.17*	0.78 ± 0.44	−51.52*
Pindone	10	9.96 ± 0.73	−0.13	10.32 ± 2.77	0.29
	100	70.55 ± 17.17	−4.20*	67.38 ± 11.70	−6.83*
Chlorophacinone	20	7.49 ± 7.32	−4.18	6.77 ± 5.78	−5.61*
	5	4.91 ± 4.94	−0.05	4.92 ± 4.90	−0.04
Bromadiolone	10	2905.30 ± 2022.44	3.51	2252.96 ± 848.49	6.48*
	20	25.02 ± 15.25	0.81	23.63 ± 12.16	0.73

[a] The calculated concentrations were compared to the true concentrations to generate test statistics. "Method 1" = collecting an average of 6 IS response ratios to calculate the QA sample's concentration. "Method 2" = calculating one IS response ratio from the average rodenticide signal and the average internal standard signal. Interpolated concentrations deemed different from the true concentration are marked with an asterisk (*), where $p < 0.002$.

with two internal standards to achieve acceptable accuracy. It is highly encouraged that future studies consider the use of more expensive deuterated rodenticides or alternative internal standards to potentially improve quantitation methods, which is suggested in a review paper by Imran *et al.*[60] Overall, this study exemplified the improved signal and reduced fragmentation of rodenticides, expanding on the possible directions of biocide detection using the DART-ToF MS as rapid screening tool both qualitatively and semi-quantitatively.

4. Conclusion

This study met the objectives to firstly confirm that DART-ToF MS is suitable as a screening tool for rodenticide detection in environmental samples and secondly collate the positive in-mode raw data mass spectral library which, when cross-referenced with the NIST/NIJ DIT, can identify rodenticides in simulated marine water contaminated with rodenticides. Results from this portion of the study successfully achieved its goal in confirming the ability of DART-ToF MS to correctly identify rodenticides prepared as blind QA samples in mixtures, differentiating each rodenticide both from other rodenticides and additional compounds, which in the current study included one herbicide and four fungicides (boscalid, picoxystrobin, quinoxyfen, metsulfuron-methyl and carbetamide). A further aim tested the use of decanoic-d3 acid and *N*-(4-hyroxyphenyl-2,3,5,6-d4) acetamide-2,2,2-d3 as internal standards for semi-quantitative analysis, which was successful for some of the rodenticides, but more research is required.

The findings in this study demonstrated the potential for environmental monitoring using DART/ToF as a robust screening tool when capitalizing on the many parameters available, such as using spectral library, ion mode, and voltage. While the internal standard method demonstrated reasonable semi-quantitative precision, the results for accuracy would require further optimization of rodenticide quantitation. The current study was aimed as a feasibility test for this emerging, rapid procedure and, as such, was not confirmed on all of the matrices that it could be applicable to (*e.g.* groundwater, surface water, seawater, soils, sands *etc.*). While it is possible that our library could apply to other matrices, new users are advised to set up in-house libraries for their specific matrices using the current study details as guidance. The individual detection limits for each rodenticide is naturally matrix dependent. Overall, the DIT and its in-house DART-ToF MS mass spectral library proved successful with similar prospects for other aqueous contaminants such as surfactants, pesticides, herbicides, and pharmaceutical personal care products (PPCPs), offering a powerful resource for environmental monitoring and emergency preparedness. Combined with the current applications in wood and ginseng species identification, DART/ToF exhibits high potential for expedited forensic preliminary screening in environmental laboratories worldwide.

Author contributions

T. Y.: writing- original draft, investigation, formal analysis, data curation, methodology. A. S. M.: software, writing- review & editing, supervision, resources. P. B.: writing- review & editing, supervision. B. R.: writing – review & editing, investigation, data curation. M. G.: data curation. R. C.: software, writing – review & editing. V. D.: resources. H. K.: method quality assessment. J. Y.: method quality assessment. D. S.: supervision, project administration, writing-review and editing, methodology, conceptualization, resources.

Conflicts of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Further data is available in the supplemental information (SI) or upon request from the author(s). The code for the NIST/NIJ DART-MS DIT can be found at **https://data.nist.gov/od/id/mds2-2448** with **https://doi.org/10.18434/mds2-2448**. The version of the code employed for this study is version 3.

Supplementary information is available. See DOI: **https://doi.org/10.1039/d5ay01447f**.

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