



Designation: D7644 – 16

Standard Test Method for Determination of Bromadiolone, Brodifacoum, Diphacinone and Warfarin in Water by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)¹

This standard is issued under the fixed designation D7644; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This procedure covers the determination of bromadiolone, brodifacoum, diphacinone and warfarin (referred to collectively as rodenticides in this test method) in water by direct injection using liquid chromatography (LC) and detected with tandem mass spectrometry (MS/MS). These analytes are qualitatively and quantitatively determined by this test method. This test method adheres to multiple reaction monitoring (MRM) mass spectrometry.

1.2 The Detection Verification Level (DVL) and Reporting Range for the rodenticides are listed in Table 1.

1.2.1 The DVL is required to be at a concentration at least 3 times below the Reporting Limit (RL) and have a signal/noise ratio greater than 3:1. Fig. 1 displays the signal/noise ratios of the primary single reaction monitoring (SRM) transitions, and Fig. 2 displays the confirmatory SRM transitions at the DVLs for the rodenticides.

1.2.2 The reporting limit was calculated from the concentration of the Level 1 calibration standard, as shown in Table 4, accounting for the dilution of a 40 mL water sample up to a final volume of 50 mL with methanol to ensure analyte solubility.

1.3 *Units*—The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the*

Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

2. Referenced Documents

2.1 ASTM Standards:²

- D1129 Terminology Relating to Water
- D1193 Specification for Reagent Water
- D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water
- D3694 Practices for Preparation of Sample Containers and for Preservation of Organic Constituents
- D3856 Guide for Management Systems in Laboratories Engaged in Analysis of Water
- D4841 Practice for Estimation of Holding Time for Water Samples Containing Organic and Inorganic Constituents
- D5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis
- E2554 Practice for Estimating and Monitoring the Uncertainty of Test Results of a Test Method Using Control Chart Techniques

2.2 Other Documents:³

- U.S. EPA publication SW-846 Test Methods for Evaluating Solid Waste, Physical/Chemical Methods

3. Terminology

3.1 Definitions:

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *detection verification level, DVL, n*—a concentration that has a signal/noise (S/N) ratio greater than 3:1 and is at least 3 times below the Reporting Limit (RL).

¹ This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.06 on Methods for Analysis for Organic Substances in Water.

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² For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

³ Available from National Technical Information Service (NTIS), U.S. Department of Commerce, 5285 Port Royal Road, Springfield, VA, 22161 or at <http://www.epa.gov/epawaste/hazard/testmethods/index.htm>.

TABLE 1 Detection Verification Level and Reporting Range

Analyte	DVL (ng/L)	Reporting Range (ng/L)
Bromadiolone	20	125-2500
Brodifacoum	20	125-2500
Diphacinone	20	125-2500
Warfarin	20	125-2500

3.2.2 *independent reference material, IRM, n*—a material of known purity and concentration obtained either from the National Institute of Standards and Technology (NIST) or other reputable supplier. The IRM shall be obtained from a different lot of material than is used for calibration.

3.2.3 *reporting limit, RL, n*—the concentration of the lowest-level calibration standard used for quantification accounting for the sample dilution.

3.2.3.1 *Discussion*—In this test method, a 40 mL sample aliquot is diluted to a 50 mL final volume after thoroughly rinsing the collection vial with methanol for quantitative transfer. In this case, the lowest calibration level of 100 ppt would allow a reporting limit of 125 ppt to be achieved.

3.2.4 *rodenticides, n*—in this test method, bromadiolone, brodifacoum, diphacinone, and warfarin collectively.

3.3 Acronyms:

3.3.1 *CCC, n*—Continuing Calibration Check

3.3.2 *IC, n*—Initial Calibration

3.3.3 *LC, n*—Liquid Chromatography

3.3.4 *LCS/LCSD, n*—Laboratory Control Sample/
Laboratory Control Sample Duplicate

3.3.5 *MeOH, n*—Methanol

3.3.6 *mM, n*—millimolar, 1×10^{-3} moles/L

3.3.7 *MRM, n*—Multiple Reaction Monitoring

3.3.8 *MS/MSD, n*—Matrix Spike/Matrix Spike Duplicate

3.3.9 *NA, adj*—Not Available

3.3.10 *ND, n*—non-detect

3.3.11 *P&A, n*—Precision and Accuracy

3.3.12 *PPB, n*—parts per billion

3.3.13 *PPT, n*—parts per trillion

3.3.14 *QA, adj*—Quality Assurance

3.3.15 *QC, adj*—Quality Control

3.3.16 *RL, n*—Reporting Limit

3.3.17 *RSD, n*—Relative Standard Deviation

3.3.18 *RT, n*—Retention Time

3.3.19 *SDS, n*—Safety Data Sheets

3.3.20 *SRM, n*—Single Reaction Monitoring

3.3.21 *SS, n*—Surrogate Standard

3.3.22 *TC, n*—Target Compound

3.3.23 μM , *n*—micromolar, 1×10^{-6} moles/L

3.3.24 *VOA, n*—Volatile Organic Analysis

4. Summary of Test Method

4.1 This is a performance based method, and modifications are allowed to improve performance.

4.2 For rodenticide analysis, samples are shipped to the lab between 0°C and 6°C and analyzed within 14 days of collection. In the lab, the samples are spiked with surrogates, quantitatively transferred to a graduated cylinder using three methanol rinses, filtered using a syringe driven filter unit, and analyzed directly by LC/MS/MS.

4.3 Bromadiolone, brodifacoum, diphacinone, warfarin, warfarin-D₅ (surrogate) and 2-bromo-4-(1,1,3,3-tetramethylbutyl)phenol (brominated octylphenol, Br-OP, surrogate) are identified by retention time and two SRM transitions. The target analytes and surrogates are quantitated using the primary SRM transitions utilizing an external calibration. The final report issued for each sample lists the concentration of bromadiolone, brodifacoum, diphacinone, warfarin, and surrogate recoveries.

5. Significance and Use

5.1 This test method has been developed by U.S. EPA Region 5 Chicago Regional Laboratory (CRL).

5.2 Bromadiolone, brodifacoum, diphacinone and warfarin are rodenticides for controlling mice, rats, and other rodents that pose a threat to public health, critical habitats, native plants and animals, crops, food and water supplies. These rodenticides also present human and environmental safety concerns. Warfarin and diphacinone are first-generation anticoagulants, while bromadiolone and brodifacoum are second-generation. The anticoagulants interfere with blood clotting, and death can result from excessive bleeding. The second-generation anticoagulants are especially hazardous for several reasons. They are highly toxic and persist a long time in body tissues. The second-generation anticoagulants are designed to be toxic in a single feeding, but time-to-death occurs in several days. This allows rodents to feed multiple times before death, leading to carcasses containing residues that may be many times the lethal dose.⁴

5.3 This test method has been investigated for use with reagent, surface, and drinking water for the selected rodenticides.

6. Interferences

6.1 Method interferences may be caused by contaminants in solvents, reagents, glassware and other apparatus producing discrete artifacts or elevated baselines. All of these materials are demonstrated to be free from interferences by analyzing laboratory reagent blanks under the same conditions as samples.

6.2 All glassware is washed in hot water with detergent and rinsed in hot water followed by distilled water. The glassware is then dried and heated in an oven at 250°C for 15 to 30

⁴ Additional information about rodenticides is available from United States Environmental Protection Agency (EPA), <http://www.epa.gov>.

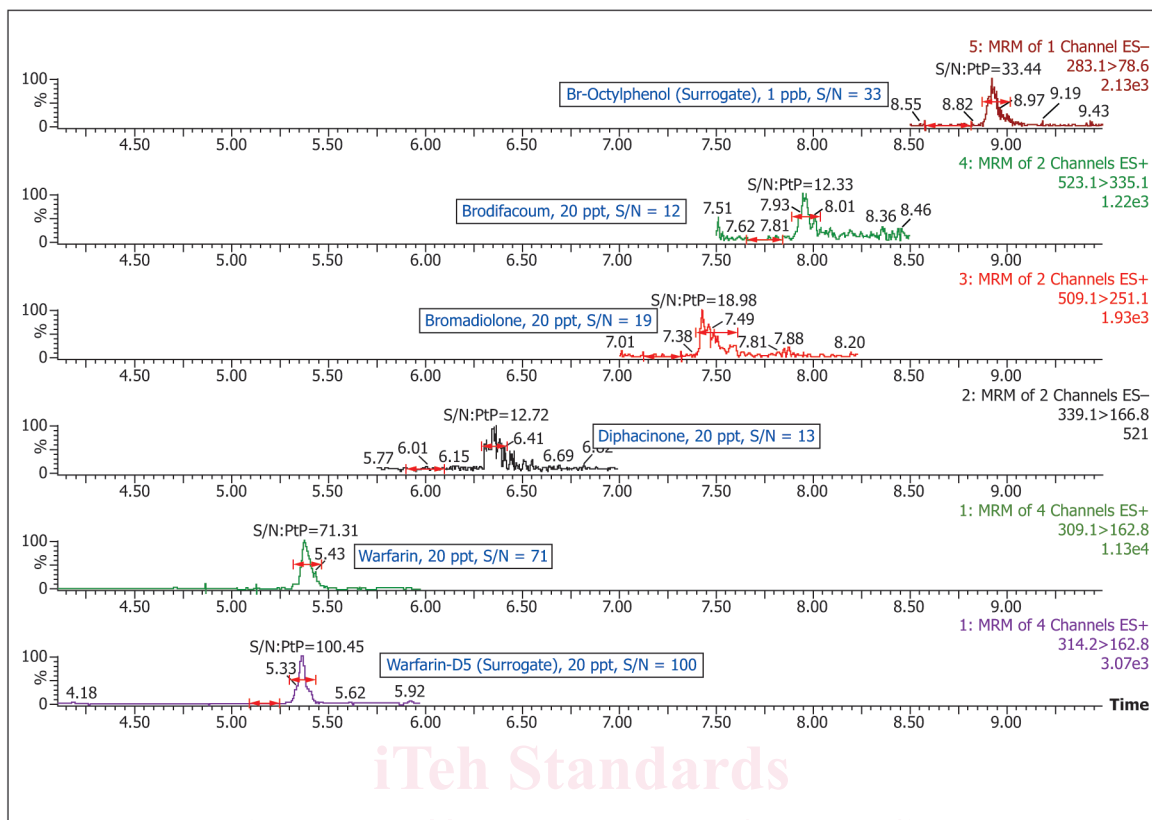


FIG. 1 Example Primary SRM Chromatograms Signal/Noise Ratios

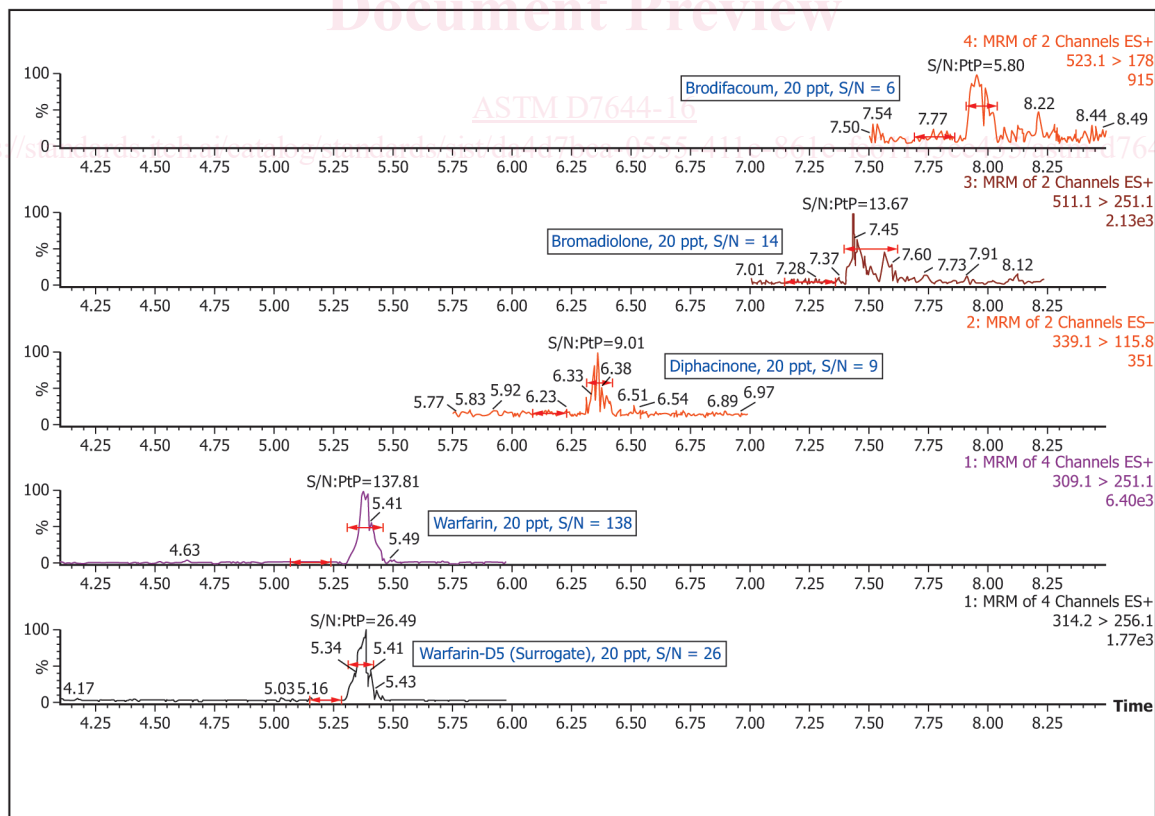


FIG. 2 Example Confirmatory SRM Chromatograms Signal/Noise Ratios

minutes. All glassware is subsequently cleaned with acetone followed by methanol.

6.3 All reagents and solvents should be of pesticide residue purity or higher to minimize interference problems.

6.4 Matrix interferences may be caused by contaminants in the sample. The extent of matrix interferences can vary considerably from sample source depending on variations of the sample matrix.

7. Apparatus

7.1 LC/MS/MS System:

7.1.1 *Liquid Chromatography (LC) System*—A complete LC system is needed to analyze samples.⁵ This should include a sample injection system, a solvent pumping system capable of mixing solvents, a sample compartment capable of maintaining required temperature and a temperature controlled column compartment. A system that is capable of performing at the flows, pressures, controlled temperatures, sample volumes, and requirements of the standard may be used.

7.1.2 *Analytical Column*⁶—A C18 column was used to develop this test method.

NOTE 1—Any column that can achieve baseline resolution of these analytes may be used. Baseline resolution simplifies data analysis and can reduce the chance of ion suppression, leading to higher limits of detection.

7.1.3 *Tandem Mass Spectrometer (MS/MS) System*—A MS/MS system capable of MRM analysis.⁷ Any system that is capable of performing at the requirements in this standard may be used.

7.2 Filtration Device:

7.2.1 *Hypodermic syringe*—A Lock Tip Glass Syringe capable of holding a syringe-driven filter unit or similar may be used.

7.2.1.1 A 50-mL lock tip glass syringe size is recommended since a 50-mL sample size is used in this test method.

7.2.2 *Filter Unit*⁸—PVDF filter units were used to filter the samples.

8. Reagents and Materials

8.1 *Purity of Reagents*—High Performance Liquid Chromatography (HPLC) pesticide residue analysis and spectrophotometry grade chemicals shall be used in all tests. Unless indicated otherwise, it is intended that all reagents shall

⁵ A Waters ACQUITY UltraPerformance Liquid Chromatography (UPLC) System (a trademark of the Waters Corporation, Milford, MA), or equivalent, was found suitable for use. All parameters in this test method are based on this system and may vary depending on your instrument.

⁶ Waters ACQUITY UPLC (a trademark of the Waters Corporation, Milford, MA) BEH C18, 2.1 × 100 mm, 1.7 μm particle size was used to develop this test method. Any column that achieves adequate resolution may be used. The retention times and order of elution may change depending on the column used and need to be monitored.

⁷ A Waters Quattro Premier (a trademark of the Waters Corporation, Milford, MA) XE tandem quadrupole mass spectrometer, or equivalent, was found suitable for use. All parameters in this test method are based on this system and may vary depending on your instrument.

⁸ A Millex HV Syringe Driven Filter Unit PVDF 0.22 μm (Millipore Corporation, Catalog #SLGV033NS; Millex is a trademark of Merck KGAA, Darmstadt, Germany) has been found suitable for use for this test method, any filter unit may be used that meets the performance of this test method may be used.

conform to the Committee on Analytical Reagents of the American Chemical Society.⁹ Other reagent grades may be used provided they are first determined to be of sufficiently high purity to permit their use without affecting the accuracy of the measurements.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Type 1 of Specification D1193. It must be demonstrated that this water does not contain contaminants at concentrations sufficient to interfere with the analysis.

8.3 *Gases*—Ultrapure nitrogen and argon.

8.4 Methanol (CAS # 67-56-1).

8.5 Acetonitrile (CAS # 75-05-8).

8.6 Acetone (CAS # 67-64-1).

8.7 Ammonium Hydroxide (Concentrated, CAS # 1336-21-6).

8.8 Ascorbic Acid (CAS # 50-81-7).

8.9 Bromadiolone (CAS # 28772-56-7).

8.10 Brodifacoum (CAS # 56073-10-0).

8.11 Diphacinone (CAS # 82-66-6).

8.12 Warfarin (CAS # 81-81-2).

8.13 Warfarin-D₅ (Phenyl-D₅, CAS # (unlabeled) 81-81-2).¹⁰

8.13.1 *Discussion*—Warfarin-D₅ is used as the electrospray positive analyte surrogate in this standard.

8.14 2-Bromo-4-(1,1,3,3-tetramethylbutyl)phenol (Br-OP).¹¹

8.14.1 *Discussion*—Br-OP is used as the electrospray negative analyte surrogate in this standard.

9. Hazards

9.1 Normal laboratory safety applies to this method. Analysts should wear safety glasses, gloves, and lab coats when working in the lab. Analysts should review the Safety Data Sheets (SDS) for all reagents used in this test method.

10. Sampling

10.1 *Sampling*—Grab samples must be collected in 40 mL pre-cleaned amber glass vials with Teflon¹²-lined caps demonstrated to be free of interferences. Surface water samples are collected unpreserved, shipped between 0°C and 6°C, and stored in the laboratory between 0°C and 6°C. Chlorinated drinking water samples are dechlorinated with ascorbic acid; 10 mg of ascorbic acid is added to each 40 mL vial prior to

⁹ Reagent Chemicals, American Chemical Society Specifications, American Chemical Society, Washington, D.C. For Suggestions on the testing of reagents not listed by the American Chemical Society, see Annual Standards for Laboratory Chemicals, BDH Ltd., Poole, Dorset, U.K., and the United States Pharmacopeia and National Formulators, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

¹⁰ A source of Warfarin-D₅ is Cambridge Isotope Laboratories, 50 Frontage Road, Andover, MA 01810-5413.

¹¹ A source of Br-OP is Accustandard, Inc., 125 Market Street, New Haven CT 06513.

¹² Teflon is a trademark of The Chemours Company in Wilmington, DE.

water collection. This test method requires a 40 mL sample size per analysis. Conventional sampling practices should be followed. Refer to Guide D3856 and Practices D3694.

10.1.1 Ammonium acetate was evaluated as an agent to bind free chlorine in drinking water and was found to be ineffective in the preservation of the rodenticides in chlorinated drinking water. Ascorbic acid was effective as a dechlorinating agent in chlorine fortified Chicago tap water, which contained 3.2 ppm free chlorine and was dechlorinated with 10 mg ascorbic acid per 40 mL water sample.¹³

10.2 The samples are collected using 40 mL glass vials. A 40 mL volume is collected directly into the sample collection vial without using any other measuring devices. This is a requirement due to the rodenticides' affinity for surfaces, which will lead to biased low results if transferring between containers. Before collection, the vials must be evaluated to determine a 40 mL sample volume. For example, the vials used in this test method were calibrated before use to determine that filling the vial to approximately 1.6 cm below the rim would result in a 40 mL sample volume. The greatest amount of water held by the 40 mL vials used in this test method was approximately 42 mL. Vials filled to 42 mL in the field would not allow the laboratory to spike the samples before quantitatively transferring to the 50 mL graduated cylinder. It is imperative that the samplers do not overfill the vials.

10.3 *Preservation*—Store samples between 0°C and 6°C from the time of collection until analysis. Analyze the sample within 14 days of collection. Chlorinated drinking water samples are dechlorinated with ascorbic acid; 10 mg of ascorbic acid is added to each 40 mL vial prior to water collection.

11. Preparation of LC/MS/MS

11.1 LC Chromatograph Operating Conditions:⁵

11.1.1 Injection volumes of all calibration standards and samples are made at 50 µL volume using a full loop injection. If a 50 µL volume loop is installed in the LC, a “full loop” mode is the preferred technique when performing fast, qualitative analyses. This mode should be used whenever accuracy and precision are the primary concerns. The first sample analyzed after the calibration curve is a blank to ensure there is no carry-over. The gradient conditions for the liquid chromatograph are shown in Table 2.

NOTE 2—If your instrument does not have a 50 µL injection capability a different volume may be used. This is a performance-based method and modifications are allowed as long as minimum performance criteria are met.

11.2 LC Sample Manager Conditions:

11.2.1 *Wash Solvents*—Weak wash is 4.0 mL of 95 % water/5 % methanol. Strong wash is 2.0 mL of methanol. The strong wash solvent is needed to eliminate carry-over between injections of rodenticide samples. The weak wash is used to remove the strong wash solvent. These rodenticides were shown to carry-over when acetonitrile was used for this

TABLE 2 Gradient Conditions for Liquid Chromatography

Time (min)	Flow (µL/min)	Percent 95% Water/ 5% Methanol, 5 mM NH ₄ OH	Percent 95% Methanol/ 5% Water, 5 mM NH ₄ OH
0.0	300	100	0
2.0	300	100	0
6.0	300	20	80
6.1	200	5	95
7.0	200	5	95
8.5	200	0	100
13.0	300	0	100
14.0	300	100	0
16.0	300	100	0

analysis. The use of methanol corrected this problem while providing separation and sensitivity. Instrument manufacturer specifications should be followed in order to eliminate sample carry-over.

11.2.2 *Temperatures*—Column, 30°C; Sample compartment, 15°C.

11.2.3 *Seal Wash*—Solvent: 50% Acetonitrile/50% Water; Time: 5 minutes.

11.3 Mass Spectrometer Parameters:⁷

11.3.1 To acquire the maximum number of data points per SRM channel while maintaining adequate sensitivity, the tune parameters may be optimized according to your instrument. Each peak requires at least 10 scans per peak for adequate quantitation. This standard contains two surrogates and four target compounds that can be acquired in 5 MRM acquisition functions. Variable parameters regarding retention times, SRM transitions, and cone and collision energies are shown in Table 3. Mass spectrometer parameters used in the development of this test method are listed below:

The instrument is set in the Electrospray source setting.

Capillary Voltage: 3.5 kV

Cone: Variable depending on analyte (Table 3)

Extractor: 2 Volts

RF Lens: 0.1 Volts

Source Temperature: 120°C

Desolvation Temperature: 375°C

Desolvation Gas Flow: 800 L/hr

Cone Gas Flow: 25 L/hr

Low Mass Resolution 1: 14.0

High Mass Resolution 1: 14.0

Ion Energy 1: 0.5

Entrance Energy: -1

Collision Energy: Variable depending on analyte (Table 3)

Exit Energy: 0

Low Mass Resolution 2: 14.0

High Mass resolution 2: 14.0

Ion Energy 2: 0.7

Multiplier: 650

Gas Cell Pirani Gauge: 7.0 × 10⁻³ Torr

Inter-Channel Delay: 0.02 seconds

Inter-Scan Delay: 0.02 seconds

Dwell: 0.1 seconds

12. Calibration and Standardization

12.1 The mass spectrometer must be calibrated per manufacturer specifications before analysis. In order to obtain valid and accurate analytical values through this test method within the confidence limits, the following procedures must be followed when performing the test method. Prepare all solutions in the lab using Class A volumetric glassware.

¹³ A Hach Pocket Colorimeter II (a trademark of Hach Company in Loveland, CO) was used to measure free chlorine.

TABLE 3 Retention Times, SRM Ions, and Analyte-Specific Mass Spectrometer Parameters

Analyte	ESI	Primary/ Confirmatory	Retention Time (min)	Cone Voltage (Volts)	Collision Energy (eV)	SRM Mass Transition (Parent > Product)	Primary/ Confirmatory SRM Area Ratio
Bromadiolone	Positive	Primary	7.45 and	30	22	509.1 > 251.1	1.0
		Confirmatory	7.59	30	22	511.1 > 251.1	
Brodifacoum	Positive	Primary	7.95	42	20	523.1 > 335.1	1.1
		Confirmatory		42	33	523.1 > 178	
Diphacinone	Negative	Primary	6.36	32	25	339.1 > 166.8	3.0
		Confirmatory		32	47	339.1 > 115.8	
Warfarin	Positive	Primary	5.38	26	14	309.1 > 162.8	1.7
		Confirmatory		26	20	309.1 > 251.1	
Warfarin-D ₅ (Surrogate)	Positive	Primary	5.37	26	14	314.2 > 162.8	1.7
		Confirmatory		26	19	314.2 > 256.1	
Br-OP (Surrogate)	Negative	Primary	8.92	35	25	283.1 > 78.6	N/A

12.2 *Calibration and Standardization*—To calibrate the instrument, analyze 7 calibration standards containing the 7 concentration levels of the rodenticides and surrogates prior to analysis as shown in Table 4. A calibration stock standard solution is prepared from standard materials or they are purchased as certified solutions. Stock standard solution A containing bromadiolone, brodifacoum, diphacinone, warfarin, warfarin-D₅ (surrogate), and 2-Bromo-4-(1,1,3,3-tetramethylbutyl)phenol (surrogate) is prepared at Level 7 concentration, and aliquots of that solution are diluted to prepare Levels 1 through 6. The following steps will produce standards with the concentration values shown in Table 4. The analyst is responsible for recording initial component weights carefully when working with pure materials and correctly carrying the weights through the dilution calculations.

12.2.1 Prepare stock standard solution A (Level 7) by adding to a 100 mL volumetric flask individual methanol solutions of the following: 50 µL of bromadiolone, brodifacoum, diphacinone, warfarin, warfarin-D₅ (surrogate) each at 4.0 mg/L and 50 µL of 2-Bromo-4-(1,1,3,3-tetramethylbutyl)phenol (surrogate) at 0.2 g/L, dilute to 100 mL with 80 % water/20 % methanol. The preparation of the Level 7 standard can be accomplished using different volumes and concentrations of stock solutions as is accustomed in the individual laboratory. Depending on the prepared stock concentrations, solubility at that concentration will have to be ensured.

12.2.2 Aliquots of solution A are then diluted with 80 % water/20 % methanol to prepare the desired calibration levels in 2 mL amber glass LC vials. The calibration vials must be used within 24 hours to ensure optimum results. Stock calibration standards are routinely replaced every 7 days if not previously discarded for quality control failure. Calibration standards are not filtered.

12.2.3 Inject each standard and obtain its chromatogram. An external calibration technique is used monitoring the primary and confirmatory SRM transition of each analyte. Calibration software is utilized to conduct the quantitation of the target analytes and surrogates using the primary SRM transition. The ratios of the primary/confirmatory SRM transition area counts are given in Table 3 and will vary depending on the individual tuning conditions. The primary/confirmatory SRM transition area ratio must be within 35 % of the individual labs accepted primary/confirmatory SRM transition area ratio. The primary SRM transition of each analyte is used for quantitation and the confirmatory SRM transition for confirmation. This gives additional confirmation by isolating the parent ion, forming two product ions via fragmentation, and relating it to the retention time in the calibration standard.

12.2.4 The calibration software manual should be consulted to properly use the software. The quantitation method is set as an external calibration using the peak areas in ppt or ppb units, as long as the analyst is consistent. Concentrations may be calculated using the data system software to generate linear regression or quadratic calibration curves. Forcing the calibration curve through the origin is not recommended.

12.2.5 Linear calibration may be used if the coefficient of determination, r^2 , is >0.98 for the analyte. The point of origin is excluded and a fit weighting of 1/X is used in order to give more emphasis to the lower concentrations. If one of the calibration standards, other than the high or low point, causes the r^2 of the curve to be <0.98, this point must be re-injected or a new calibration curve must be regenerated. If the low or high point is excluded, minimally a five point curve is acceptable but the reporting range must be modified to reflect this change.

12.2.6 Quadratic calibration may be used if the coefficient of determination, r^2 , is >0.99 for the analyte. The point of origin is excluded, and a fit weighting of 1/X is used in order

TABLE 4 Concentrations of Calibration Standards (ppt)

Analyte/Surrogate	LV 1	LV 2	LV 3	LV 4	LV 5	LV 6	LV 7
Bromadiolone	100	200	500	750	1000	1500	2000
Brodifacoum	100	200	500	750	1000	1500	2000
Diphacinone	100	200	500	750	1000	1500	2000
Warfarin	100	200	500	750	1000	1500	2000
Warfarin-D ₅ (Surrogate)	100	200	500	750	1000	1500	2000
Br-OP (Surrogate)	5000	10 000	25 000	37 500	50 000	75 000	100 000