Designation: ~~D7644 – 10ε2~~ 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.

~~ε[1] NOTE—This test method was corrected editorially in April 2011.~~
~~ε[2] NOTE—This test method was corrected editorially in February 2012.~~

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 and health practices and determine the applicability of regulatory limitations prior to use.*

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

[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.

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

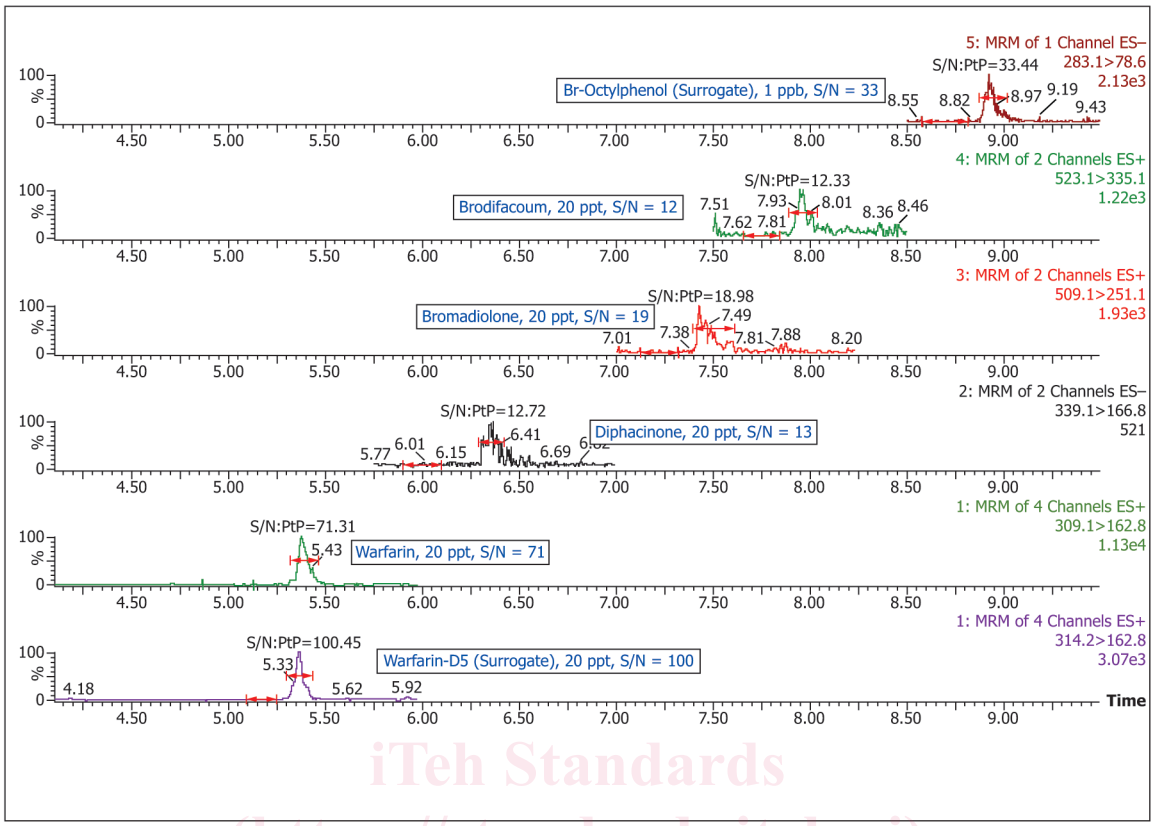

FIG. 1 Example Primary SRM Chromatograms Signal/Noise Ratios

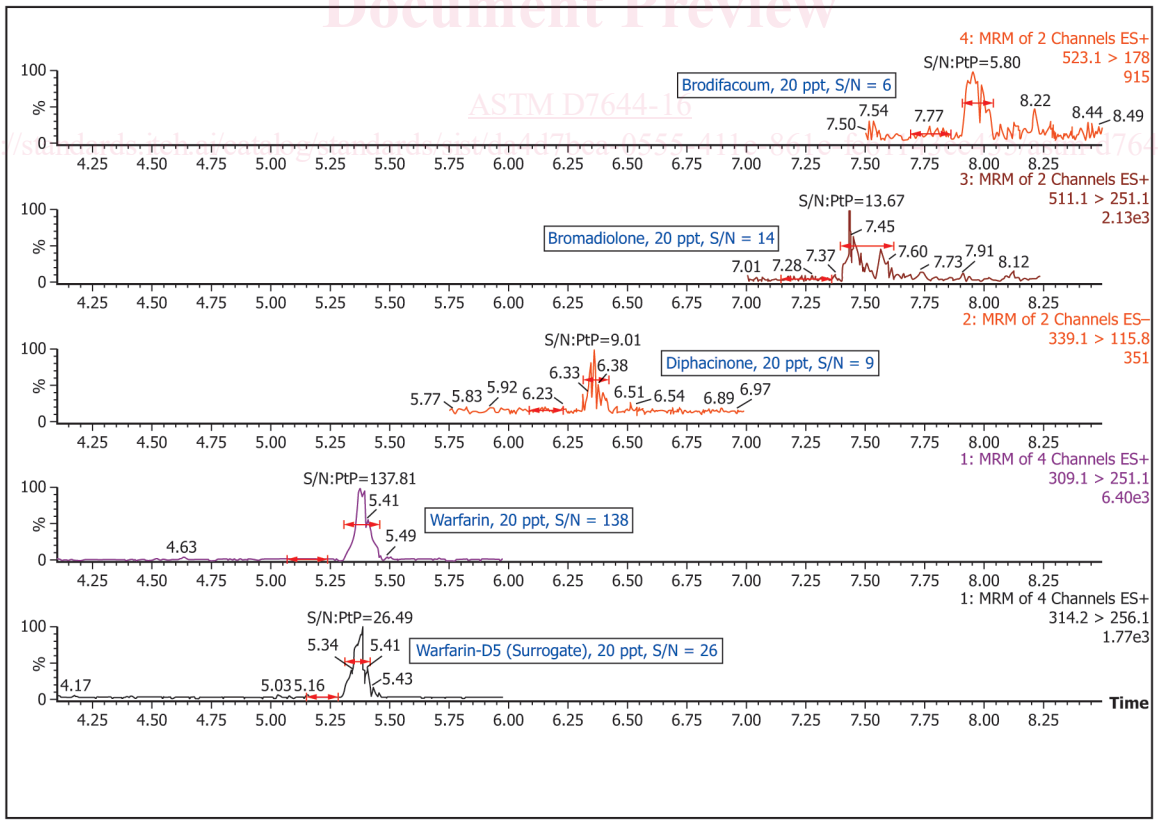

FIG. 2 Example Confirmatory SRM Chromatograms Signal/Noise Ratios

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:*~~*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).
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] 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.

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 ~~*Abbreviations:*~~*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—*~~*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—*~~*ND, n*—non-detect
3.3.11 *P&A, n*—Precision and Accuracy
3.3.12 ~~*ppt—*~~*PPB, n*—parts per ~~trillion, ng/L~~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-D_5 (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 ~~US~~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 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] ~~Any system~~ 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–Waters—~~Column*[6] ~~—ACQUITY UPLC® BEH C18, 2.1 × 100 mm, 1.7 µm particle size~~ A C18 column 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.~~

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.

[4] Additional information about rodenticides ~~can be found on the internet at http://www.epa.gov (2010).~~is available from United States Environmental Protection Agency (EPA), http://www.epa.gov.

[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 ~~ACQUITY UltraPerformance Liquid Chromatography (UPLC®) System was used to develop this test method.~~ 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.

7.2 *Filtration Device:*

7.2.1 *Hypodermic ~~Syringe—~~syringe—*A Lock Tip Glass Syringe capable of holding a ~~Millex® HV Syringe Driven Filter Unit PVDF 0.22 µm~~ syringe-driven filter unit or similar may be used.

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

7.2.2 ~~*Filter—*~~*Filter Unit*[8] ~~—Millex® HV Syringe Driven Filter Unit PVDF 0.22 µm (Millipore Corporation, Catalog # SLGV033NS) or similar may be used.~~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 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-D_5 (Phenyl-D_5, CAS # (unlabeled) 81-81-2).[10]

8.13.1 *Discussion*—Warfarin-D_5 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 ~~Material~~ Safety Data Sheets ~~(MSDS)~~(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 ~~Teflon®~~Teflon[12] ~~lined~~ -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 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.

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[8] A ~~Waters Quattro Premier™ XE tandem quadrupole mass spectrometer was used to develop this test method. All parameters in this test method are based on this system and may vary depending on your instrument.~~ 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.

[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-D_5 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.

TABLE 2 Gradient Conditions for Liquid Chromatography

Time (min)	Flow (µL/min)	Percent 95% Water/ 5% Methanol, 5 mM NH_4OH	Percent 95% Methanol/ 5% Water, 5 mM NH_4OH
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

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 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

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