

**Analytical method for difenacoum in soil**

**Reports:** ECM: EPA MRID No.: 50623628. Morlacchini, M. 2006. Residues Determination of Brodifacoum, Difenacoum and Bromadiolone in Soil – Final Report for Difenacoum Residue Determination. Study ID: CZ/05/002/ACTIVA/SOIL. Document ID: CZ/05/002/ACTIVA/SOIL/RF\_DIFENACOUM. Report prepared by CERZOO, Centro Ricerche per la Zootecnia e l’Ambiente, Piacenza, sponsored and submitted by “Difenacoum & Brodifacoum Task Force” (Activa Srl, Milano, Italy, and Pelgar International Ltd, Hampshire, United Kingdom); 47 pages (erratically numbered and unnumbered). Draft report issued October 21, 2005; Revisions 1, 2 3, 4, and 5 issued October 28, 2005, November 24, 2005, December 21, 2005, January 4, 2006, and January 13, 2006, respectively.

ILV: EPA MRID No. 50623627. Marshall, L. 2009. Validation of a Method for the Determination of Difenacoum Residues in Sediment. Study ID: CEMR/4470; Study No.: CEMS-4470. Report prepared by CEM Analytical Services Limited (CEMAS), Berkshire, United Kingdom, sponsored and submitted by Pelgar International Ltd, Hampshire, United Kingdom, and Activa Srl, Milano, Italy; 43 pages (including 1A). Final report issued November 24, 2009.

**Document No.:** MRIDs 50623628 & 50623627




**Guideline:** 850.6100

**Statements:** ECM: The study was conducted in accordance with Directive n.88/320/CEE and n.90/18/CEE Good Laboratory Practice (GLP) standards and the Italian Department of Health [p. 12; Annex 2, pp. 29-30 (written in Italian) of MRID 50623628]. Signed and dated No Data Confidentiality, Quality Assurance, and GLP statements were provided (pp. 2, 12; Annex 2, pp. 29-30). Authenticity statement was not included. A Summary of Revisions to Previous Versions was not included.

ILV: The study was conducted in accordance with the OECD and UK GLP standards and the UK Department of Health (p. 2; Appendix 5, p. 42 of MRID 50623627). Signed and dated No Data Confidentiality, GLP and Quality Assurance statements were provided (pp. 1A-2, 4; Appendix 5, p. 42). A certification of authenticity was included with the Quality Assurance statement.

**Classification:** This analytical method is classified as unacceptable. Two sets of performance data were not submitted for either the HPLC/UV (CERZOO) or LC/MS/MS (CEMAS) method. An updated ECM should be submitted which incorporates the ILV modifications and validates the LOQ of the ILV. The specificity of the ILV method was not supported by representative chromatograms. In the ECM, no samples were prepared at the LOQ, and no confirmation method was included. The reviewer could not find a domestic address for either of the study sponsors/submitters. ECM and ILV matrices were not insufficiently characterized. The number of ILV trials required to validate the method was not reported. The LOQs of the ECM and ILV

differed significantly since the analytical methods differed.

<b>PC Code:</b>	119901		
<b>EFED Final Reviewer:</b>	Andrew Shelby, Physical Scientist	Signature:	
		Date:	June 23, 2020
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		Date:	11/07/2018

*This Data Evaluation Record may have been altered by the Environmental Fate and Effects Division subsequent to signing by CDM/CSS-Dynamac JV personnel. The CDM/CSS-Dynamac Joint Venture role does not include establishing Agency policies.*

## Executive Summary

The analytical method, CERZOO Study CZ/05/002/ACTIVA/SOIL, is designed for the quantitative determination of difenacoum in soil at the LOQ of 0.252 µg/g using HPLC/UV. The analytical method, CEMAS Study ID CEMR/4470 [CEMAS SOP CEM-3440 (draft)], is designed for the quantitative determination of difenacoum in sediment at the LOQ of 0.01 µg/g using LC/MS. The LOQs of the ECM and ILV differed significantly since the analytical methods differed. The LOQ of the HPLC/UV (CERZOO) is less than the lowest toxicological level of concern in soil based on lowest rat acute LD50 (male acute oral LD50 = 1.8 mg/kg-bw, MRID 46750935), soil incorporation to one inch, and soil bulk density of once gram per cubic centimeter. Two sets of performance data were not submitted for either the HPLC/UV (CERZOO) or LC/MS/MS (CEMAS) method. It appeared that the CEMAS method was a modification of the CERZOO method in which significant modifications were made at the request of the Sponsor to eliminate the use of chloroform in the extraction. **An updated ECM should be submitted which incorporates the ILV modifications and validates the LOQ of the ILV.** The ECM and ILV used one soil or sediment matrix each, but matrices were not insufficiently characterized. No confirmation method was included in the HPLC/UV (CERZOO) method; a confirmation method is usually required when LC/MS or GC/MS is not used as the primary method to generate study data. Two ion transitions were monitored in the LC/MS/MS (CEMAS) method. All ILV and ECM data regarding repeatability, accuracy, precision, and linearity were satisfactory; however, no samples were prepared at the LOQ in the ECM. The specificity of the ILV method was not supported by representative chromatograms because significant peak shouldering occurred. The number of ILV trials required to validate the method was not reported.

**Table 1. Analytical Method Summary**

Analyte(s) by Pesticide	MRID		EPA Review	Matrix	Method Date (dd/mm/yyyy)	Registrant	Analysis	Limit of Quantitation (LOQ)
	Environmental Chemistry Method	Independent Laboratory Validation						
Difenacoum	50623628	None submitted		Soil <sup>1,2</sup>	21/10/2005 (Draft)	Pelgar International Ltd, and Activa Srl,	HPLC/UV	0.252 µg/g
					28/10/2005 (Revision 1)			
24/11/2005 (Revision 2)								
21/12/2005 (Revision 3)								
04/01/2006 (Revision 4)								
13/01/2006 (Revision 5)								
	None submitted	50623627		Sediment	24/11/2009		LC/MS/MS	0.01 µg/g

1 In the ECM, the soil (25.84% silt, 26.52% clay, 47.64% sand; pH 5.95; 5.04% organic matter) was used in the study (p. 16 of MRID 50623628). Soil characterization was not reported as USDA soil texture classification; soil texture was not identified in the study. Soil sources were not described.

2 In the ILV, the river sediment collected from Quelm Lane, Bracknell, was used in the study (p. 10 of MRID 50623627). Soil/sediment characterization was not provided; soil/sediment texture was not identified in the study.

**Page numbers referenced for ECM MRID 50623628 were based on the pdf document page since the pages of the study report appeared to be a compilation of data which contained many non-numbered pages and independently numbered portions. Identifying study report section titles were reported with the page numbers whenever possible.**

## I. Principle of the Method

### ECM

Soil (40.0 g) in 500-mL soxhlet was fortified with difenacoum in acetone for procedural recoveries (pp. 16, 18 of MRID 50623628). The samples were extracted three times (100 mL, 100 mL, and 50 mL sequentially) with acetone:chloroform (50:50, v:v) via mechanical shaker (*ca.* 180 rpm for 30 minutes). After filtration on glass fiber, extract was collected in a 500-mL rotavapor balloon. The solvent of the combined extracts was evaporated at 200 mm of Hg. The residue was reconstituted in 10 mL of acetone and purified in a glass column with 6 g of florisil

and 1 g of anhydrous sodium sulphate. After being applied to the column, the column was washed with 40 mL of acetone, and the eluate was collected in a flask. The acetone solvent was evaporated with nitrogen. The residue was reconstituted with 1 mL of methanol:water (1:1, v:v). After centrifugation (5 minutes at 200 rpm), the final solution was transferred to a 2-mL vial for HPLC analysis. If not analyzed immediately, the sample was stored in a freezer at -20°C.

Samples were analyzed using an Agilent™ HPLC 1100 binary pump with Diode Array Detector (DAD) detector (pp. 16-17 of MRID 50623628). The following conditions were used: Synergy 4µ Fusion RP80A Phenomenex column (150 x 4.60 mm, particle size not reported; S/N 224016-2; column temperature 25°C), gradient mobile phase of A) water with 0.1% formic acid and B) acetonitrile [time, percent A:B; 0.00-5.00 min. 50.0:50.0, 10.00-15.00 min. 10.0:90.0, 18.00-23.00 min. 50.0:50.0], and UV detection (264 nm). Injection volume was 20 µL. Expected retention time for difenacoum was *ca.* 12.75 minutes. No confirmation method was reported.

### ILV

The ILV reportedly performed CEMAS SOP CEM-3440 (draft) “Analytical Method for the Determination of Difenacoum in Sediment” not CERZOO Study CZ/05/002/ACTIVA/SOIL (p. 11; Appendix 4, pp. 28-29 of MRID 50623627). The extraction of the soil/sediment was modified from three times with acetone:chloroform (50:50, v:v) to once with acetone:hexane (80:20, v:v; 100 mL). The extract was purified using a Waters Oasis MAX SPE cartridge (150 mg, 6 cc) and eluted with ethyl acetate:methanol:formic acid (90:8:2, v:v:v; 22 mL) instead of the florisil column eluted with acetone. The SPE column was pre-conditioned sequentially with methanol, water, methanol, ethyl acetate, methanol, and 5% ammonium in water (5 mL each). The extract was mixed with 100 µL of ammonium prior to application to the SPE column. After concentration of the eluate, the final residue was reconstituted in acetonitrile:water (80:20, v:v; 5 mL) instead of methanol:water (1:1, v:v). The final sample was filtered (0.45 µm) or diluted, as necessary. The reviewer considered the ILV method to be a significantly modified version of the ECM.

Samples were analyzed using an Agilent™ HPLC 1100 binary pump coupled with an Applied Biosystems MDS SCIEX API 5000 mass spectrometer (Appendix 4, pp. 30-31 of MRID 50623627). The following conditions were used: Luna Phenyl-Hexyl column (150 x 3.0 mm, particle size 3 µm; column temperature 30°C), gradient mobile phase of A) 10 mM ammonium acetate and B) HPLC grade acetonitrile [time, percent A:B; 0.0-0.5 min. 80:20, 5.0-7.5 min. 10:90, 7.6-9.0 min. 80:20], and Turbo Spray in negative polarity and MRM scan. Injection volume was 20 µL. Expected retention time for difenacoum was 6.0 minutes. Two ion transitions were monitored (quantitation and confirmation, respectively):  $m/z$  443.10 → 135.20 and  $m/z$  443.10 → 293.10.

### LOQ/LOD

In the ECM, the Limit of Quantification (LOQ) for difenacoum in CERZOO Study CZ/05/002/ACTIVA/SOIL was reported as 0.252 µg/g using HPLC/UV (pp. 13, 23 of MRID 50623628). The Limit of Detection (LOD) for difenacoum was 0.006 µg/g. In the ILV, the LOQ for difenacoum in CEMAS Study ID CEMR/4470 [CEMAS SOP CEM-3440 (draft)] was

reported as 0.01 µg/g using LC/MS (pp. 8-9 of MRID 50623627). The LOD for difenacoum was 0.020 ng/mL for the quantitation ion transition and 0.27 ng/mL for the confirmation ion transition. The LOQs of the ECM and ILV differed significantly since the analytical methods differed.

## II. Recovery Findings

ECM (MRID 50623628): For CERZOO Study CZ/05/002/ACTIVA/SOIL, mean recoveries and relative standard deviations (RSD) were within guideline requirements (mean 70-120%; RSD ≤20%) for analysis of difenacoum at the 0.016 µg/g (*ca.* 0.06×LOQ), 0.063 µg/g (0.25×LOQ), and 0.158 µg/g (*ca.* 0.63×LOQ) in one soil matrix (Table 5, p. 21; DER Attachment 2). No samples were prepared at the LOQ, 0.252 µg/g. HPLC/UV was used as the primary analysis, but no confirmation method was included in the method. A confirmation method is usually required when LC/MS or GC/MS is not used as the primary method to generate study data. Means, standard deviations, and RSDs were reviewer-calculated based on data provided in the study report since the study author did not report these values (see DER Attachment 2). Rules of significant figures were followed. The soil (25.84% silt, 26.52% clay, 47.64% sand; pH 5.95; 5.04% organic matter) was used in the study (p. 16). Soil characterization was reported as USDA soil texture classification; soil texture was not identified in the study. Soil sources were not described.

ILV (MRID 50623627): For CEMAS Study ID CEMR/4470 [CEMAS SOP CEM-3440 (draft)], mean recoveries and RSDs were within guidelines for analysis of difenacoum at the LOQ (0.01 µg/g) and 10×LOQ (0.1 µg/g) in one sediment matrix (Tables 4-5, pp. 13-14; DER Attachment 2). Two ion transitions were monitored via LC/MS analysis; performance data (results) were comparable for the quantitation and confirmation ion transition. The recovery results were corrected for residues quantified in the controls; Appendix 4, p. 32). The river sediment collected from Quelm Lane, Bracknell, was used in the study (p. 10). Soil/sediment characterization was not provided; soil/sediment texture was not identified in the study. The ILV was reportedly performed to validate CEMAS SOP CEM-3440 (draft) “Analytical Method for the Determination of Difenacoum in Sediment” not CERZOO Study CZ/05/002/ACTIVA/SOIL; however, it appeared that the CEMAS method was a modification of the CERZOO method in which modifications were made at the request of the Sponsor to eliminate the use of chloroform in the extraction (p. 11; Appendix 4, pp. 28-29). The number of trials was not reported; however, based on all of the information provided in the ILV, the reviewer considered the ILV method to be a significantly modified version of the ECM which the ILV validated in the first trial after an incomplete trial of the original ECM. **An updated ECM should be submitted** which incorporates the ILV modifications and validates the LOQ of the ILV modified method.

**Table 2. Initial Validation Method Recoveries for Difenacoum in Soil<sup>1,2</sup>**

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%) <sup>3</sup>	Relative Standard Deviation (%)
<b>Soil</b>						
Primary Method – HPLC/UV						
Difenacoum	0.016	4	89.3-90.0	89.7	0.4	0.4
	0.063	4	94.9-95.1	95.0	0.1	0.1
	0.158	4	88.6-92.7	91.6	2.0	2.2
	0.252 (LOQ)	--	<b>No samples prepared</b>			
Confirmation Method - <b>None</b>						

Data (uncorrected recovery results; pp. 18-19) were obtained from Table 5, p. 21 of MRID 50623628 and DER Attachment 2.

- 1 The soil (25.84% silt, 26.52% clay, 47.64% sand; pH 5.95; 5.04% organic matter) was used in the study (p. 16). Soil characterization was reported as USDA soil texture classification; soil texture was not identified in the study. Soil sources were not described.
- 2 Means, standard deviations, and RSDs were reviewer-calculated based on data provided in the study report since the study author did not report these values (see DER Attachment 2). Rules of significant figures were followed.

**Table 3. Independent Validation Method Recoveries for Difenacoum in Sediment<sup>1,2</sup>**

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%) <sup>3</sup>	Relative Standard Deviation (%)
<b>Sediment</b>						
LC/MS/MS						
Quantitation ion transition						
Difenacoum	0.01 (LOQ)	5	90-101	98	5	4.9
	0.1	5	108-109	108	0	0.5
Confirmation ion transition						
Difenacoum	0.01 (LOQ)	5	82-105	92	10	10.5
	0.1	5	104-107	105	1	1.3

Data (recovery results were corrected for residues quantified in the controls; Appendix 4, p. 32) were obtained from Tables 4-5, pp. 13-14 of MRID 50623627 and DER Attachment 2.

- 1 The river sediment collected from Quelm Lane, Bracknell, was used in the study (p. 10). Soil characterization was not provided; soil texture was not identified in the study.
- 2 Difenacoum was identified using two ion transitions were monitored (quantitation and confirmation, respectively):  $m/z$  443.10 → 135.20 and  $m/z$  443.10 → 293.10.
- 3 Standard deviations were reviewer-calculated based on data provided in the study report since the study author did not report these values (see DER Attachment 2). Rules of significant figures were followed.

### III. Method Characteristics

In the ECM, the LOQ and LOD for difenacoum in CERZOO Study CZ/05/002/ACTIVA/SOIL were reported as 0.252 µg/g and 0.006 µg/g, respectively, using HPLC/UV (pp. 13, 23 of MRID 50623628). Following the general method of Keith, L. H., *et al.* (not identified or referenced), the LOD and LOQ for determination of difenacoum in soil were calculated using the standard deviation from the 0.64 µg/g recovery results. The LOD was calculated as three times the standard deviation (3s), and the LOQ was calculated as ten times the standard deviation (10s) of the recovery results. The calculated LOQ was 0.0214 µg/g. Since this value was below the lowest standard, the study author reported 0.252 µg/g, which was the value of the lowest standard.

In the ILV, the LOQ and LOD for difenacoum in CEMAS Study ID CEMR/4470 [CEMAS SOP CEM-3440 (draft)] was reported as 0.01 µg/g using LC/MS (pp. 8-9; Appendix 4, p. 35 of MRID 50623627). The LOQ was defined as the lowest fortification level at which acceptable recovery data are obtained (recovery in the range of 70 to 110%). The LOQ was not justified with calculations or comparisons to background noise. Detection limits should not be based on the arbitrarily selected lowest concentration in the spiked samples. The LOD was 0.020 ng/mL for the quantitation ion transition and 0.27 ng/mL for the confirmation ion transition. The LOD was calculated using the following equation:

$$\text{LOD} = [4 \times \text{Noise} / (h_{\text{peak}})] \times c_{\text{standard}}$$

Where, LOD is the limit of detection, Noise is the background noise height,  $h_{\text{peak}}$  is the peak height, and  $c_{\text{standard}}$  is the concentration of the low calibration standard.

The LOQs of the ECM and ILV differed significantly since the analytical methods differed.

**Table 4. Method Characteristics for Difenacoum in Soil**

Analyte		Difenacoum	
Analysis		HPLC/UV	LC/MS/MS <sup>1</sup>
Limit of Quantitation (LOQ)	ECM	0.252 µg/g	-- <sup>2</sup>
	ILV	--	0.01 µg/g
Limit of Detection (LOD)	ECM	0.006 µg/g,	--
	ILV	--	0.020 ng/mL (Q) 0.27 ng/mL (C)
Linearity (calibration curve r <sup>2</sup> and concentration range)	ECM	r <sup>2</sup> = 0.99999	--
	ILV	--	r <sup>2</sup> = 0.9999 (Q) r <sup>2</sup> = 0.9993 (C)
	Range	0.252-12.6 µg/g	0.001-0.05 µg/mL
Repeatable	ECM <sup>3</sup>	<b>No samples were prepared at the LOQ.</b> Yes at <i>ca.</i> 0.06×LOQ, 0.25×LOQ, and <i>ca.</i> 0.63×LOQ in one soil matrix.	--
	ILV <sup>4,5</sup>	--	Yes at LOQ and 10×LOQ in one sediment matrix.
Reproducible	<b>No at LOQ and 10×LOQ.</b> Two sets of performance data were not submitted for either the HPLC/UV or LC/MS/MS method.		
Specific	ECM	Yes, no matrix interferences were observed. <b>No confirmation method was performed.</b> <sup>6</sup>	
	ILV	<b>No</b> , matrix interferences were measured as <i>ca.</i> 2% of the LOQ (based on peak height); however, significant peak shouldering occurred at the LOQ and 10×LOQ which significantly interfered with peak integration and attenuation.	

Data were obtained from pp. 13, 23 (LOQ/LOD); Table 5, p. 21 (recovery results); Table 4, p. 19; Figure 3, p. 20 (calibration data & curve); Annex 4, pp. 40-47 (chromatograms) of MRID 50623628; pp. 8-9; Appendix 4, p. 35 (LOQ/LOD); Table 4-5, pp. 13-14 (recovery results); p. 16; Appendix 1, pp. 18-19 (calibration curves); Appendix 2, pp. 20-23 (chromatograms) of MRID 50623627; DER Attachment 2.

1 Difenacoum was identified in the ILV using two ion transitions were monitored (quantitation and confirmation, respectively):  $m/z$  443.10 → 135.20 and  $m/z$  443.10 → 293.10.

2 Not performed.

3 In the ECM, the soil (25.84% silt, 26.52% clay, 47.64% sand; pH 5.95; 5.04% organic matter) was used in the study (p. 16 of MRID 50623628). Soil characterization was not reported as USDA soil texture classification; soil texture was not identified in the study. Soil sources were not described.

4 In the ILV, the river sediment collected from Quelm Lane, Bracknell, was used in the study (p. 10 of MRID 50623627). Soil/sediment characterization was not provided; soil/sediment texture was not identified in the study.

5 The ILV was reportedly performed to validate CEMAS SOP CEM-3440 (draft) "Analytical Method for the Determination of Difenacoum in Sediment" not CERZOO Study CZ/05/002/ACTIVA/SOIL; however, it appeared that the CEMAS method was a modification of the CERZOO method in which modifications were made at the request of the Sponsor to eliminate the use of chloroform in the extraction (p. 11; Appendix 4, pp. 28-29). The number of trials was not reported; however, based on all of the information provided in the ILV, the reviewer considered the ILV method to be a significantly modified version of the ECM which the ILV validated in the first trial after an incomplete trial of the original ECM. An updated ECM should be submitted which incorporates the ILV modifications and validates the LOQ of the ILV modified method.

6 A confirmation method is usually required when LC/MS or GC/MS is not used as the primary method to generate study data.



#### IV. Method Deficiencies and Reviewer's Comments

1. Two sets of performance data were not submitted for either the HPLC/UV (CERZOO) or LC/MS/MS (CEMAS) method. OCSPP guidelines state that two sets of performance data should be submitted, one for the initial or other internal validation and one for the ILV.
2. An updated ECM should be submitted which incorporates the ILV modifications and validates the LOQ of the ILV. The ILV was reportedly performed to validate CEMAS SOP CEM-3440 (draft) "Analytical Method for the Determination of Difenacoum in Sediment" not the ECM method, CERZOO Study CZ/05/002/ACTIVA/SOIL; however, it appeared that the CEMAS method was a modification of the CERZOO method in which modifications were made at the request of the Sponsor to eliminate the use of chloroform in the extraction (p. 11; Appendix 4, pp. 28-29 of MRID 50623627). The ILV reported that data was collected using the acetone:chloroform (50:50, v:v) extraction solution, but this data was not reported in the ILV. The data provided in the draft CEMAS method match the data provided in the ILV study report, so only one set of performance data was submitted in the ILV study report. Based on all of the information provided in the ILV, the reviewer considered the ILV method to be a significantly modified version of the ECM which the ILV validated in the first trial after an incomplete trial of the original ECM.
3. The specificity of the ILV method was not supported by representative chromatograms because significant peak shouldering occurred at the LOQ and 10×LOQ which significantly interfered with peak integration and attenuation (Appendix 2, pp. 20-23 of MRID 50623627).
4. In the ECM, no samples were prepared at the LOQ, and no confirmation method was included. OCSPP guidelines state that minimum of five spiked replicates were analyzed at each concentration (*i.e.*, minimally, the LOQ and 10× LOQ) for each analyte. Also, a confirmation method is usually required when LC/MS or GC/MS is not used as the primary method to generate study data.
5. The reviewer could not find a domestic address for either of the study sponsors/submitters, Activa Srl (Milano, Italy) and Pelgar International Ltd (Hampshire, United Kingdom).
6. The ECM and ILV matrices were not insufficiently characterized. The soil texture characterization of the ECM soil was reported, but characterization was reported as USDA soil texture classification; soil texture was not identified in the study (25.84% silt, 26.52% clay, 47.64% sand; pH 5.95; 5.04% organic matter) was used in the study (p. 16 of MRID 50623628). Soil. Soil sources were not described. The ILV soil/sediment characterization was not provided; soil/sediment texture was not identified in the study (p. 10 of MRID 50623627).
7. The number of ILV trials required to validate the method was not reported.

8. The LOQs of the ECM and ILV differed significantly since the analytical methods differed.
9. The estimations of the LOQ and LOD in the ILV were not based on scientifically acceptable procedures as defined in 40 CFR Part 136 (pp. 8-9; Appendix 4, p. 35 of MRID 50623627). The LOQ was defined as the lowest fortification level at which acceptable recovery data are obtained (recovery in the range of 70 to 110%). The LOQ was not justified with calculations or comparisons to background noise. Detection limits should not be based on the arbitrarily selected lowest concentration in the spiked samples. The LOD was calculated using the following equation:  $LOD = [4 \times \text{Noise} / (h_{\text{peak}})] \times c_{\text{standard}}$ , where, LOD is the limit of detection, Noise is the background noise height,  $h_{\text{peak}}$  is the peak height, and  $c_{\text{standard}}$  is the concentration of the low calibration standard.
10. In the ILV, the matrix effects were determined to be insignificant ( $< \pm 20\%$ ; p. 16 of MRID 50623627). Solvent standards were used.
11. The time required to complete the method was not reported in the ECM or ILV.

## V. References

- U.S. Environmental Protection Agency. 2012. Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation. Office of Chemical Safety and Pollution Prevention, Washington, DC. EPA 712-C-001.
- 40 CFR Part 136. Appendix B. Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11, pp. 317-319.

**Attachment 1: Chemical Names and Structures****Difenacoum**

**IUPAC Name:** 3-(3-Biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin

**CAS Name:** Not reported

**CAS Number:** 56073-07-5

**SMILES String:** Not found

