Test Material:	Methoxyfenozide
MRID:	49525704
Title:	Method Validation Study for the Determination of Methoxyfenozide and Its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Soil and Sediment Using Liquid Chromatography with Tandem Mass Spectrometry
MRID:	49525701
Title:	Methoxyfenozide and its Metabolites - Independent Laboratory Validation of the Method for the Determination of Residues of Methoxyfenozide in Soil and Sediment by LC-MS/MS
EPA PC Code:	121027
OCSPP Guideline:	850.6100

For CDM Smith

Primary Reviewer: Lisa Muto **Signature:** 

Date: 10/20/15

Secondary Reviewer: Kathleen Ferguson **Signature:**

Date: 10/20/15

QC/QA Manager: Joan Gaidos **Signature:** 

Date: 10/20/15

Analytical method for methoxyfenozide (RH-2485) and its transformation products, A-ring phenol metabolite of methoxyfenozide and B-ring mono acid metabolite of methoxyfenozide (RH-131154), in soil and sediment

Reports: ECM: EPA MRID No.: 49525704. Shackelford, D.D. and M.J. Walter. 2014. Method Validation Study for the Determination of Methoxyfenozide and Its A-ring Phenol Metabolite and B-ring Mono Acid Metabolite in Soil and Sediment Using Liquid Chromatography with Tandem Mass Spectrometry. Laboratory Study ID: 110354. Report prepared, sponsored and submitted by Regulatory Sciences and Government Affairs, Dow AgroSciences LLC, Indianapolis, Indiana; 97 pages. Final report issued June 23, 2014.
ILV: EPA MRID No. 49525701. Jones, S. 2012. Methoxyfenozide and its Metabolites - Independent Laboratory Validation of the Method for the Determination of Residues of Methoxyfenozide in Soil and Sediment by LC-MS/MS. EAS Study No.: S11-04019. Dow AgroSciences Study Reference No.: 110756. Report prepared by Eurofins Agroscience Services (EAS) Ltd., Derbyshire, United Kingdom, and sponsored and submitted by Dow AgroSciences LLC, Indianapolis, Indiana; 134 pages. Final report issued August 15, 2012.

Document No.: MRIDs 49525704 & 49525701

Guideline: 850.6100

Statements: ECM: The statement of Good Laboratory Practice (GLP) standards could not read because the copy of the page was too faint (p. 3 of MRID 49525704). Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided (the QA page was faint but readable; pp. 2-4). A statement of the authenticity of the study report was included with the quality assurance statement (p. 4).
ILV: The study was conducted in accordance with USEPA and OECD GLP standards (1998), as well as the UK Department of Health (soil characterization; p. 3; Appendix B, Appendix 2, p. 111 of MRID 49525701). Signed and dated No Data Confidentiality, GLP, Quality Assurance and Authenticity statements were provided (pp. 2-4; Appendix B, Appendix 2, p. 111). A statement of the authenticity of the study report was included with the quality assurance statement (p. 4).

Classification: This analytical method is classified supplemental. In the ECM, the number of samples was insufficient for all analyses. ECM representative chromatograms were inadequate to support the method; also, a reagent blank was not included. The linearity coefficient (r^2) of the B-ring mono acid metabolite was not always ≥ 0.995.

` 121027

Reviewer: Karen Milians, Chemist, Ph.D.

Signature:

Date: Digitally signed by KAREN MILIANS
DN: c=US, o=U.S. Government, ou=USEPA, ou=Staff, cn=KAREN MILIANS, dnQualifier=0000048934
Date: 2016.11.15 13:46:51 -05'00'

All page numbers refer to those listed in the upper right-hand corner of the MRIDs.

Executive Summary

The analytical method, Method Validation No.110354, is designed for the quantitative determination of methoxyfenozide (RH-2485), A-ring phenol metabolite of methoxyfenozide (RH-2485; N'-(3-hydroxy-2-methylbenzoyl)-N-(3,5-dimethylbenzoyl)-N-*t*-butyl hydrazine) and B-ring mono acid metabolite of methoxyfenozide (RH-131154; (3-({1 *tert*-butyl-2-[(3-methoxy-2-methylphenol)carbonyl]hydrazinyl}-carbonyl)-5-methylbenzoic acid)) in soil and sediment at the LOQ of 0.01 µg/g using LC/MS/MS. The LOQ is less than the lowest toxicological level of concern in soil/sediment for all analytes. The ECM method was validated by the ILV in the first trial. In the ECM, the number of samples was insufficient for all analyses, and representative chromatograms from only two of the four matrices were included to support the method.

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						
Methoxy-fenozide	49525704	49525701		Soil[1,2]	23/06/2014	Dow AgroSciences LLC	LC/MS/MS	0.01 mg/kg
A-ring Phenol Metabolite								
B-ring Mono Acid Metabolite								

1 For ECM, clay loam soil (29% sand, 39% silt, 32% clay; 2.2% organic carbon), silt loam soil (10% sand, 65% silt, 25% clay; 2.7% organic carbon), loamy sand sediment (85% sand, 13% silt, 2% clay; 5.8% organic carbon) and sand sediment (89% sand, 10% silt, 1% clay; 6.4% organic carbon) were used in the study (USDA classifications were reported; p. 13; Table 2, p. 25 of MRID 49525704).

2 For the ILV, loamy sand soil (78% and, 17% silt, 5% clay; 1.5% organic carbon) and clay sediment (2% sand, 36% silt, 62% clay; 1.6% organic carbon) were used for validation (USDA texture classification; p. 13; Appendix B, pp. 100-101, 112-113 of MRID 49525701).

I. Principle of the Method

Samples (5.0 ± 0.05 g) of soil and sediment in 45-mL vials equipped with PTFE-lined caps or 50-mL polypropylene graduated centrifuge tubes equipped with caps were fortified, as necessary, then extracted twice with methanol:0.1N hydrochloric acid (90:10, v:v; 20 mL then 15 mL) via vortex mixing (*ca*. 10 seconds) and shaking on a reciprocating flat-bed shaker for at least 30 minutes at *ca*. 80 excursions/minute (p. 12; Appendix 1, pp. 93-94 of MRID 49525704). The method noted that it was critical that the soil/sediment plug was broken-up prior to placing the samples on the flat-bed shaker. After centrifugation (*ca.* 5 minutes at *ca.* 2000 rpm), both extracts were combined in a graduated mixing cylinder or 50-mL polypropylene graduated centrifuge tube equipped with cap, then the volume was adjusted to 40.0 mL with methanol:0.1N hydrochloric acid (90:10, v:v). After mixing, an aliquot of the extract (1.0 mL) was diluted with water (2.0 mL) in a 8-mL vial and vortexed for *ca*. 5 seconds. The aliquot was purified using offline reversed-phase extraction (SPE) procedure (Strata-X polymeric sorbent reversed-phase SPE cartridge; 30-mg, 1-mL). The SPE column was pre-conditioned with methanol and water (1 mL each) with full vacuum (*ca*. -15 to -25 in Hg). The 1.0 sample aliquot was applied to the column (*ca*. 1 mL/min rate); the eluate was discarded. The column was washed with 1.0 mL of water:methanol:formic acid (60:40:0.1, v:v:v). After drying the column with vacuum, the analytes were eluted with two 75-µL aliquots of acetonitrile under full vacuum (*ca*. 1 mL/min rate). The purified sample was evaporated to dryness under nitrogen on a TurboVap evaporator at *ca*. 40°C and reconstituted with 1.0 mL of water:acetonitrile containing 0.1% formic acid (70:30, v:v). The final solution was analyzed by liquid chromatography using positive-ion electrospray ionization (ESI) with tandem mass spectrometry.

Samples were analyzed for methoxyfenozide and its metabolites using an AB/Sciex API 4000 LC/MS/MS (p. 14; Appendix 1, pp. 96-97 of MRID 49525704). The instrumental conditions consisted of a Gemini C18 110A column (2.00 x 50 mm, 5-µm; column temperature ambient, *ca*. 22°C), SecurityGuard cartridge for Gemini C18 HPLC column with 2.0 to 3.0 ID, a mobile phase gradient of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid [percent A:B (v:v) at 0.0-1.00 min. 70:30, 8.00 min. 10:90, 9.00-12.00 min. 70:30], MS/MS detection in positive turbo spray (MRM; temperature, 350°C), and injection volume 30 µL. Two parent-daughter ion transitions were monitored per analyte (quantification and confirmation, respectively): *m/z* 369.1 → 313.2 and *m/z* 369.1 → 149.2 for methoxyfenozide, *m/z* 355.0 → 299.2 and *m/z* 355.0 → 135.2 for A-ring phenol, and *m/z* 399.1 → 343.1 and *m/z* 399.1 → 149.2 for B-ring mono acid. Retention times were observed at *ca*. 5.1, 3.9, and 3.95 min. for methoxyfenozide, A-ring phenol and B-ring mono acid, respectively (Figures 23-25, pp. 79-81; Figures 27-29, pp. 83-85).

ILV

In the ILV, the ECM was performed exactly as written, except for the use of different LC/MS/MS conditions (pp. 14-15 of MRID 49525701). Samples were analyzed for methoxyfenozide and its metabolites using an Applied Biosystems API 4000 LC/MS/MS equipped with an Ascentis Express C18 column (2.1 x 50 mm, 2.7-µm; column temperature 40°C; pp. 16-18). The mobile phase gradient was (A) water containing 0.1% formic acid and (B) acetonitrile [percent A:B (v:v) at 0.0-1.00 min. 70:30, 8.00 min. 10:90, 9.00-12.00 min. 70:30]

and MS/MS detection in positive turbo spray (MRM; temperature, 500°C), and injection volume 15 µL. These modifications of the LC/MS/MS conditions were minor changes and approved by the Study Director. Two parent-daughter ion transitions were monitored per analyte (quantification and confirmation, respectively): *m/z* 369.2 → 313.3 and *m/z* 369.2 → 149.1 for methoxyfenozide, *m/z* 355.1 → 299.2 and *m/z* 355.1 → 135.0 for A-ring phenol, and *m/z* 399.3 → 343.1 and *m/z* 399.3 → 149.2 for B-ring mono acid. Retention times were observed at *ca*. 4.5, 3.05 and 3.15 min. for methoxyfenozide, A-ring phenol and B-ring mono acid, respectively (Figures 21-22, pp. 48-49; Figures 27-28, pp. 54-55; Figures 33-34, pp. 60-61).

LOQ/LOD

The LOQ for all analytes was the same in the ECM and ILV at 0.01 µg/g (0.01 mg/kg; pp. 12, 17; Table 27, p. 46 of MRID 49525704; pp. 12, 21 of MRID 49525701). The LOD for all analytes was 0.003 µg/g in the ECM; the LOD was not reported in the ILV.

II. Recovery Findings

ECM (MRID 49525704): Mean recoveries and relative standard deviations (RSDs) were within guidelines for analysis of methoxyfenozide (RH-2485), A-ring phenol metabolite of methoxyfenozide and B-ring mono acid metabolite of methoxyfenozide (RH-131154) in two soils (clay loam and silt loam) and two sediments (loamy sand and sand) at fortification levels of 0.01 µg/g (LOQ) and 1.00 µg/g (100×LOQ); however, the number of samples was insufficient for all analyses (n = 3; quantitative and confirmatory HPLC analyses; Tables 9-20, pp. 32-43). At the 0.10 µg/g (10×LOQ) fortification level, the number of samples was also insufficient for all analyses (n = 2); individual recoveries were satisfactory (70-120%) for analysis of all three analytes in the four test matrices (quantitative and confirmatory HPLC analyses). Mean recoveries and relative standard deviations (RSDs) of statistical significance could not be calculated for 10×LOQ. Performance data (recovery results) of the quantitative HPLC analysis and confirmatory HPLC analysis were comparable. The ECM calculations allowed for recovery data to be corrected for residues found in the control samples; however, residues were not quantified in any of the control samples (Tables 9-20, pp. 32-43; Figures 10-15, pp. 66-71). Recoveries from samples fortified at 0.003 µg/g (LOD) ranged (ions/matrices combined) from 57-82% for methoxyfenozide, 43-74% for A-ring phenol metabolite and 61-96% for B-ring mono acid metabolite (n = 1 for each matrix/analyte; DER Attachment 2). The soil/sediment matrices were well characterized (USDA classifications; p. 13; Table 2, p. 25). Clay loam soil (110354-005; 29% sand, 39% silt, 32% clay; 2.2% organic carbon) from California, USA; silt loam soil (110354-007; 10% sand, 65% silt, 25% clay; 2.7% organic carbon) from Iowa, USA; and loamy sand sediment (110354-006; 85% sand, 13% silt, 2% clay; 5.8% organic carbon) and sand sediment (110354-008; 89% sand, 10% silt, 1% clay; 6.4% organic carbon) from Swiss Lake, Derbyshire, UK were used in the study.

ILV (MRID 49525701): Mean recoveries and RSDs were within guidelines for analysis of methoxyfenozide, A-ring phenol metabolite and B-ring mono acid metabolite in loamy sand soil and clay sediment at fortification levels of 0.01 µg/g (LOQ) and 0.10 µg/g (10×LOQ; quantitative and confirmatory HPLC analyses; Tables 1-6, pp. 24-26). Performance data (recovery results) of the quantitative HPLC analysis and confirmatory HPLC analysis were comparable; the only noted difference was for performance data of B-ring mono acid metabolite in clay sediment at the LOQ. Loamy sand soil (78% and, 17% silt, 5% clay; 1.5% organic carbon) from Germany and clay sediment (2% sand, 36% silt, 62% clay; 1.6% organic carbon) from another EAS study (source not further specified) were used for validation (USDA texture classification; p. 13; Appendix B, pp. 100-101, 112-113). The method was validated in the first trial (p. 20).

Table 2. Initial Validation Method Recoveries for Methoxyfenozide and Its Metabolites, A-ring Phenol and B-ring Mono Acid, in Two Soils and Two Sediments[1,2]

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Clay Loam Soil (California, -005)						
	Quantitation ion					
Methoxyfenozide	0.003 (LOD)	1	79	--	--	--
	0.010 (LOQ)	3	85-89	87	2	2
	0.10	2	94, 98	--	--	--
	1.00	3	92-93	93	1	1
A-ring Phenol Metabolite	0.003 (LOD)	1	74	--	--	--
	0.010 (LOQ)	3	86-88	87	1	1
	0.10	2	93, 96	--	--	--
	1.00	3	90-93	91	2	2
B-ring Mono Acid Metabolite	0.003 (LOD)	1	86	--	--	--
	0.010 (LOQ)	3	79-93	87	7	8
	0.10	2	90, 93	--	--	--
	1.00	3	91-94	92	2	2
	Confirmation ion					
Methoxyfenozide	0.003 (LOD)	1	82	--	--	--
	0.010 (LOQ)	3	85-90	88	3	3
	0.10	2	93, 97	--	--	--
	1.00	3	92-93	92	1	1
A-ring Phenol Metabolite	0.003 (LOD)	1	55	--	--	--
	0.010 (LOQ)	3	80-85	82	3	4
	0.10	2	92, 96	--	--	--
	1.00	3	90-92	91	1	1
B-ring Mono Acid Metabolite	0.003 (LOD)	1	81	--	--	--
	0.010 (LOQ)	3	81-94	86	7	8
	0.10	2	88, 90	--	--	--
	1.00	3	90-92	91	1	1
Silt Loam Soil (Iowa, -007)						
	Quantitation ion					
Methoxyfenozide	0.003 (LOD)	1	59	--	--	--
	0.010 (LOQ)	3	85-95	90	5	6
	0.10	2	94, 96	--	--	--
	1.00	3	88-91	90	2	2
A-ring Phenol Metabolite	0.003 (LOD)	1	55	--	--	--
	0.010 (LOQ)	3	80-91	84	6	7
	0.10	2	91, 93	--	--	--
	1.00	3	86	86	0	0
B-ring Mono Acid Metabolite	0.003 (LOD)	1	89	--	--	--
	0.010 (LOQ)	3	86-95	89	5	6
	0.10	2	88, 90	--	--	--
	1.00	3	85-88	87	2	2
	Confirmation ion					
Methoxyfenozide	0.003 (LOD)	1	57	--	--	--
	0.010 (LOQ)	3	85-96	90	6	6

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
	0.10	2	95, 97	--	--	--
	1.00	3	88-91	89	2	2
A-ring Phenol Metabolite	0.003 (LOD)	1	43	--	--	--
	0.010 (LOQ)	3	83-90	87	4	4
	0.10	2	90, 94	--	--	--
	1.00	3	86	86	0	0
B-ring Mono Acid Metabolite	0.003 (LOD)	1	91	--	--	--
	0.010 (LOQ)	3	87-91	89	2	2
	0.10	2	88	--	--	--
	1.00	3	85-87	86	1	1
Loamy Sand Sediment (Swiss Lake, United Kingdom, -006)						
	Quantitation ion					
Methoxyfenozide	0.003 (LOD)	1	66	--	--	--
	0.010 (LOQ)	3	91-94	93	2	2
	0.10	2	100, 103	--	--	--
	1.00	3	92-96	94	2	2
A-ring Phenol Metabolite	0.003 (LOD)	1	67	--	--	--
	0.010 (LOQ)	3	89-97	93	4	4
	0.10	2	99, 104	--	--	--
	1.00	3	92-94	93	1	1
B-ring Mono Acid Metabolite	0.003 (LOD)	1	75	--	--	--
	0.010 (LOQ)	3	90-93	92	2	2
	0.10	2	95, 96	--	--	--
	1.00	3	91-94	93	2	2
	Confirmation ion					
Methoxyfenozide	0.003 (LOD)	1	68	--	--	--
	0.010 (LOQ)	3	94-95	95	1	1
	0.10	2	98, 101	--	--	--
	1.00	3	92-95	94	2	2
A-ring Phenol Metabolite	0.003 (LOD)	1	73	--	--	--
	0.010 (LOQ)	3	92-96	94	2	2
	0.10	2	98, 103	--	--	--
	1.00	3	93-95	94	1	1
B-ring Mono Acid Metabolite	0.003 (LOD)	1	61	--	--	--
	0.010 (LOQ)	3	85-93	89	4	5
	0.10	2	91, 92	--	--	--
	1.00	3	91-92	92	1	1
Sand Sediment (Swiss Lake, United Kingdom, -008)						
	Quantitation ion					
Methoxyfenozide	0.003 (LOD)	1	70	--	--	--
	0.010 (LOQ)	3	91-94	92	2	2
	0.10	2	95, 98	--	--	--
	1.00	3	91-93	92	1	1
A-ring Phenol Metabolite	0.003 (LOD)	1	63	--	--	--
	0.010 (LOQ)	3	85-94	92	5	6
	0.10	2	95, 98	--	--	--

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
	1.00	3	91-92	92	1	1
B-ring Mono Acid Metabolite	0.003 (LOD)	1	87	--	--	--
	0.010 (LOQ)	3	87-99	93	6	7
	0.10	2	89, 93	--	--	--
	1.00	3	91	91	0	0
	Confirmation ion					
Methoxyfenozide	0.003 (LOD)	1	74	--	--	--
	0.010 (LOQ)	3	91-95	93	2	2
	0.10	2	97, 99	--	--	--
	1.00	3	91-93	92	1	1
A-ring Phenol Metabolite	0.003 (LOD)	1	70	--	--	--
	0.010 (LOQ)	3	88-91	90	2	2
	0.10	2	96, 99	--	--	--
	1.00	3	91-92	92	1	1
B-ring Mono Acid Metabolite	0.003 (LOD)	1	96	--	--	--
	0.010 (LOQ)	3	90-106	98	8	8
	0.10	2	88, 91	--	--	--
	1.00	3	90-91	90	1	1

The numbers of test in red represent less number of samples than recommended and recovery range in red represent lower % than acceptable levels).

Data (uncorrected recovery results; Tables 9-20, pp. 32-43; Figures 10-15, pp. 66-71) were obtained from Tables 9-20, pp. 32-43 of MRID 49525704 and DER Attachment 2 (means, s.d. and RSDs at LOQ and 100×LOQ, and % recovery at LOD). Recovery statistics were reviewer-calculated because reported recovery statistics were only provided for combined soil matrices and combined sediment matrices.

1 The soil matrices were well characterized (USDA classifications were reported; p. 13; Table 2, p. 25). Clay loam soil (110354-005; 29% sand, 39% silt, 32% clay; 2.2% organic carbon) from California, USA; silt loam soil (110354-007; 10% sand, 65% silt, 25% clay; 2.7% organic carbon) from Iowa, USA; and loamy sand sediment (110354-006; 85% sand, 13% silt, 2% clay; 5.8% organic carbon) and sand sediment (110354-008; 89% sand, 10% silt, 1% clay; 6.4% organic carbon) from Swiss Lake, Derbyshire, UK were used in the study.

2 Two parent-daughter ion transitions were monitored per analyte (quantification and confirmation, respectively): *m/z* 369.1 → 313.2 and *m/z* 369.1 → 149.2 for methoxyfenozide, *m/z* 355.0 → 299.2 and *m/z* 355.0 → 135.2 for A-ring phenol, and *m/z* 399.1 → 343.1 and *m/z* 399.1 → 149.2 for B-ring mono acid (p. 14; Appendix 1, pp. 96-97).

Table 3. Independent Validation Method Recoveries for Methoxyfenozide and Its Metabolites, A-ring Phenol and B-ring Mono Acid, in Soil and Sediment[1,2]

Analyte	Fortification Level (µg/g)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Loamy Sand Soil						
	Quantitation ion					
Methoxyfenozide	0.01 (LOQ)	5	93-101	99	3	3.3
	0.1	5	87-95	92	3	3.7
A-ring Phenol Metabolite	0.01 (LOQ)	5	91-100	96	4	3.7
	0.1	5	90-100	95	4	3.8
B-ring Mono Acid Metabolite	0.01 (LOQ)	5	89-94	92	4	3.9
	0.1	5	88-96	92	3	3.1
	Confirmation ion					
Methoxyfenozide	0.01 (LOQ)	5	96-102	98	3	2.9
	0.1	5	90-97	93	3	2.9
A-ring Phenol Metabolite	0.01 (LOQ)	5	91-99	93	3	3.6
	0.1	5	87-93	91	3	2.9
B-ring Mono Acid Metabolite	0.01 (LOQ)	5	92-101	96	4	4.4
	0.1	5	89-97	93	4	3.8
Clay Sediment						
	Quantitation ion					
Methoxyfenozide	0.01 (LOQ)	5	86-100	93	6	6.1
	0.1	5	88-96	92	3	3.5
A-ring Phenol Metabolite	0.01 (LOQ)	5	88-100	94	6	5.9
	0.1	5	89-95	91	2	2.7
B-ring Mono Acid Metabolite	0.01 (LOQ)	5	71-85	78	5	6.5
	0.1	5	71-75	73	2	2.5
	Confirmation ion					
Methoxyfenozide	0.01 (LOQ)	5	88-95	92	4	3.8
	0.1	5	90-96	92	3	2.8
A-ring Phenol Metabolite	0.01 (LOQ)	5	88-100	94	5	5.7
	0.1	5	88-94	90	2	2.8
B-ring Mono Acid Metabolite	0.01 (LOQ)	5	64-77	71	6	8.4
	0.1	5	67-74	70	3	3.7

Data (uncorrected results; Appendix C, p. 114) were obtained from Tables 1-6, pp. 24-26 of MRID 49525701 and DER Attachment 2 (s.d. and RSDs at LOQ and 10×LOQ).

1 Loamy sand soil (78% and, 17% silt, 5% clay; 1.5% organic carbon) from Germany and clay sediment (2% sand, 36% silt, 62% clay; 1.6% organic carbon) from another EAS study (source not further specified) were used for validation (USDA texture classification; p. 13; Appendix B, pp. 100-101, 112-113).

2 Two parent-daughter ion transitions were monitored per analyte (quantification and confirmation, respectively): *m/z* 369.2 → 313.3 and *m/z* 369.2 → 149.1 for methoxyfenozide, *m/z* 355.1 → 299.2 and *m/z* 355.1 → 135.0 for A-ring phenol, and *m/z* 399.3 → 343.1 and *m/z* 399.3 → 149.2 for B-ring mono acid (p. 17).

III. Method Characteristics

In the ECM and ILV, the LOQ value for methoxyfenozide and its metabolites, A-ring phenol and B-ring mono acid, was established at 0.01 µg/g (0.01 mg/kg; pp. 12, 17, 22; Table 27, p. 46 of MRID 49525704; pp. 12, 21 of MRID 49525701). The LOD for all analytes in the ECM was 0.003 µg/g. The LOD was not reported in the ILV. Following the method of Keith, L. H., *et al*. (see section **V. References** below), the LOD and LOQ for determination of methoxyfenozide and its metabolites in soil/sediment were calculated in the ECM using the standard deviation from the 0.010 µg/g recovery results. The LOD was calculated as three times the standard deviation (3*s*), and the LOQ was calculated as ten times the standard deviation (10*s*) of the recovery results. The calculated values support the LOQ and LOD established for the study and are presented in **Table 4** below.

Table 4. Method Characteristics

		Methoxyfenozide	A-ring phenol metabolite	B-ring mono acid metabolite
Limit of Quantitation (LOQ)	Established	0.01 µg/g		
	Calculated (ECM)	0.00158-0.00428 µg/g	0.00328-0.00434 µg/g	0.00392-0.00719 µg/g
Limit of Detection (LOD)	Established	0.003 µg/g		
	Calculated (ECM)	0.000474-0.00128 µg/g	0.000985-0.00130 µg/g	0.00118-0.00216 µg/g
Linearity (Least squares calibration curve r and concentration range)	ECM[1]	r^2 = 0.99834 (Q) r^2 = 0.99792 (C)	r^2 = 0.99845 (Q) r^2 = 0.99845 (C)	r^2 = 0.99993 (Q) r^2 = 0.99992 (C)
		0.10-75 ng/mL		
	ILV[2]	r^2 = 0.9994-0.9996 (Q) r^2 = 0.9996-0.9998 (C)	r^2 = 0.9998 (Q) r^2 = 0.9998-1.0000 (C)	r^2 = **0.9924**-1.0000 (Q) r^2 = 0.9998 (C)
		0.0001-0.01 µg/mL		
Repeatable	ECM[3]	Yes at LOQ and 100×LOQ, but n = **3**. Yes at 10×LOQ, but n = **2**.		
	ILV[4]	Yes at LOQ and 10×LOQ (n = 5).		
Reproducible		Yes at the LOQ and 10×LOQ.		
Specific	ECM	Only chromatograms of silt loam soil (007) and sand sediment (008) were provided.		
		Yes, no interferences were observed in the matrix control.		
	ILV	Yes, no interferences were observed in the matrix control. A minor contaminant (RT 3.3-3.4 min.) was observed in the confirmation ion spectra of the sediment matrix samples; extremely minor peak integration interferences were noted for B-ring metabolite due to the peak.		

Numbers in red represent less than acceptable values.
Data were obtained from pp. 12, 17, 22; Tables 9-20, pp. 32-43; Table 27, p. 46; Figures 4-9, pp. 60-65; Figures 19-29, pp. 75-85 of MRID 49525704; pp. 12, 21; Tables 1-6, pp. 24-26; Figures 1-13, pp. 28-40; Figures 17-52, pp. 44-79 of MRID 49525701. Q = Quantitative HPLC analysis; C = Confirmatory HPLC analysis.
1 ECM standard curves were weighted 1/x. ECM r^2 values are reviewer-generated for all analytes from reported r values of 0.9991721-0.9999638 (Q) and 0.99896191-0.9999622 (C; calculated from data in Figures 4-9, pp. 60-65 of MRID 49525704; see DER Attachment 2).
2 ILV standard curves were weighted 1/x. ILV r^2 values are reviewer-generated for all analytes from reported r values of 0.9998-1.000 (Q- Soil), 0.9962-1.000 (Q- Sediment), 0.9998-0.9999 (C- Soil) and 0.9999-1.000 (C- Sediment; calculated from data in Figures 1-13, pp. 28-40 of MRID 49525701; see DER Attachment 2).
3 For the ECM, clay loam soil (29% sand, 39% silt, 32% clay; 2.2% organic carbon), silt loam soil (10% sand, 65% silt, 25% clay; 2.7% organic carbon), loamy sand sediment (85% sand, 13% silt, 2% clay; 5.8% organic carbon) and sand sediment (89% sand, 10% silt, 1% clay; 6.4% organic carbon) were used in the study (USDA classifications were reported; p. 13; Table 2, p. 25 of MRID 49525704).
4 For the ILV, loamy sand soil (78% and, 17% silt, 5% clay; 1.5% organic carbon) and clay sediment (2% sand, 36% silt, 62% clay; 1.6% organic carbon) were used for validation (USDA texture classification; p. 13; Appendix B, pp. 100-101, 112-113 of MRID 49525701).
Linearity is satisfactory when $r^2 \geq 0.995$.

IV. Method Deficiencies and Reviewer's Comments

1. In the ECM, the number of samples was insufficient for all analyses at the LOQ and 100×LOQ (n = 3), and at 10×LOQ (n = 2; Tables 9-20, pp. 32-43 of MRID 49525704). OSCPP guidelines recommend a minimum of five samples spiked at each fortification level.

2. In the ECM, representative chromatograms were only provided for sand sediment (008) and silt loam soil (007; Figures 19-29, pp. 75-85 of MRID 49525704). OCSPP guidelines recommend that chromatograms be provided for all matrices which were included in the validation. Also, a reagent blank was not included.

3. The linearity coefficient (r^2) of the B-ring mono acid metabolite was not always ≥ 0.995 (Figures 11-12, pp. 38-39 of MRID 49525701 and DER Attachment 2). The reviewer-calculated r^2 value was 0.9924 for the LOQ set.

4. The ILV soil/sediment matrices were loamy sand soil (5% clay; 1.5% organic carbon) and clay sediment (62% clay; 1.6% organic carbon; p. 13; Appendix B, pp. 100-101, 112-113 of MRID 49525701).

5. The toxicological level of concern was not reported for the analytes in soil/sediment. A LOQ above toxicological levels of concern results in an unacceptable method classification.

6. The reviewer noted that the titling of the confirmation chromatograms of the analytes contained "qualifier(IS)" versus "qualifier" in the quantification chromatograms (Figures 17-52, pp. 44-79 of MRID 49525701). The reviewer noted that the "IS" did not refer to an internal standard.

7. The ILV study reported that communications between the ILV performing laboratory and the sponsor were unnecessary (p. 20 of MRID 49525701).

8. In the ECM, the stability of the working solutions was assessed based on the results of a previous study [Method Validation Study No. 110356 (MRID 49525703; Shackelford, D.D., and M.J. Walter. 2014); pp. 17-20, 23; Tables 28-35, pp. 47-53 of MRID 49525704]. It was determined that the calibration standards (water:acetonitrile containing 0.1% formic or acetic acid, 70:30, v:v) were stable for at least 135-141 days when protected from light under ambient conditions. The sample extracts from soil and sediment were stable for up to 15 days under refrigeration storage (*ca*. 4°C).

   In the ECM, matrix effects were also studied (p. 20; Tables 36-41, pp. 54-56 of MRID 49525701). Matrix effects were insignificant (±5%) for all matrices.

9. It was reported for the ILV that the analytical procedure for one set of 15 samples required approximately 7.5 person hours for preparation (p. 20 of MRID 49525701). The LC/MS/MS was conducted overnight unattended. The interpretation of data required approximately 3 hours. The overall time to complete a set of samples was 1.5 calendar days.

V. References

Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* 1983, *55*, 2210-2218 (p. 22 of MRID 49525704).

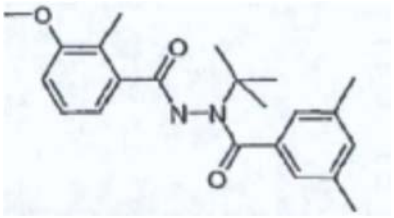
U.S. Environmental Protection Agency. 2012. Ecological Effects Test Guidelines, OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation. Office of Chemical Safety and Pollution Prevention, Washington, DC. EPA 712-C-001.

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

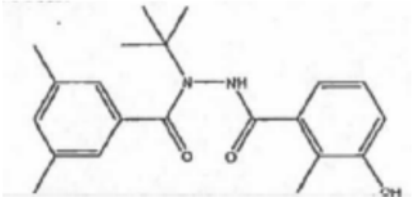
Methoxyfenozide (RH-2485; RH-112485)

IUPAC Name: Not reported
CAS Name: 3-Methoxy-2-methylbenzoic acid 2-(3,5-dimethylbenzoyl)-2-(1,1-dimethylethyl)hydrazide
CAS Number: 161050-58-4
SMILES String: Not found



A-ring Phenol Metabolite of RH-2485

IUPAC Name: Not reported
CAS Name: N'-(3-hydroxy-2-methylbenzoyl)-N-(3,5-dimethylbenzoyl)-N-t-butyl hydrazine
CAS Number: 252720-16-4
SMILES String: Not found



B-ring Mono Acid Metabolite of Methoxyfenozide (RH-131154)

IUPAC Name: Not reported
CAS Name: (3-({1-tert-butyl-2-[(3-methoxy-2-methylphenol)carbonyl]hydrazinyl}-carbonyl)-5-methylbenzoic acid)
CAS Number: Not found
SMILES String: Not found