

Test Material: Tioxazafen/MON 102100

MRID: 49304239

Title: Analytical Method for the Determination of MON 102100 and Its Major Metabolite Benzamidine in Soil

MRID: 49304240

Title: Independent Laboratory Validation of an Analytical Method for the Determination of MON 102100 and Benzamidine in Soil

EPA PC Code: 074752

OCSPP Guideline: 850.7100

For CDM Smith

Primary Reviewer: Lisa Muto

Signature: 

Date: 8/18/15

Secondary Reviewer: Kathleen Ferguson

Signature:

Date: 8/18/15

QC/QA Manager: Joan Gaidos

Signature: 

Date: 8/18/15

Secondary Reviewer: Christopher M. Koper

Date: 09/01/15

Chemist, EPA/OCSPP/OPP/EFED/ERB1



Analytical method for MON 102100 (tioxazafen) and its transformation product benzamidine in soil

- Reports:** ECM: EPA MRID No. 49304239. Riter, L.S., C.E. Wujcik, P.K. Jensen. 2014. Analytical Method for the Determination of MON 102100 and Its Major Metabolite Benzamidine in Soil. Method Name: High Throughput Assay for MON 102100 and Benzamidine in Soil (Appendix 1, p. 30). Report No.: MSL0025796. Method No.: AG-ME-1636-03 (Appendix 1, p. 30). Report prepared, sponsored and submitted by Monsanto Company, St. Louis, Missouri; 56 pages. Method date August 29, 2014 (Appendix 1, p. 30). Final report issued November 11, 2014.
ILV: EPA MRID No. 49304240. Bendler, S.E. 2014. Independent Laboratory Validation of an Analytical Method for the Determination of MON 102100 and Benzamidine in Soil. EPL BAS Study No.: 115G805. Monsanto Study ID No.: EBS-2014-0100 and Report No.: MSL0025798. Report prepared by EPL Bio Analytical Services (EPL BAS), Niantic, Illinois, sponsored and submitted by Monsanto Company, St. Louis, Missouri; 152 pages (including 2 non-paginated pages). Final report issued November 12, 2014.
- Document No.:** MRIDs 49304239 & 49304240
- Guideline:** 850.6100
- Statements:** ECM: The study was conducted in accordance with USEPA FIFRA Good Laboratory Practice (GLP) standards (p. 3 of MRID 49304239). Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided (pp. 2-5).
ILV: The study was conducted in accordance with USEPA FIFRA GLP standards (p. 3 of MRID 49304240). Signed and dated No Data Confidentiality, GLP, and Quality Assurance statements were provided (pp. 2-5).
- Classification:** This analytical method is **scientifically sound** and classified as **Supplemental**. The ILV was not provided with the most up-to-date version of the method in order to perform the independent validation. The determination of the LOQ and LOD were not based on scientifically acceptable procedures. For MON 102100 in the ECM, performance data to validate the method at 10×LOQ were not reported. The ECM report did not provide adequate and accurate chromatograms to support the method. A reagent blank was not included in the ECM. In the ECM pre-study validation, recovery results were corrected and soil matrices were not characterized or described. ECM MRID 49304239 did not provide raw data or recovery ranges for any of the validation study.

All page numbers refer to those listed in the upper right-hand corner of the MRIDs, with the exception that Page 1 of MRID 49304240 was written at the bottom center of the page.

Some data was taken from the terrestrial field dissipation study MRID 49304217 (see References); data was cited based on the page numbers on the lower right-hand side of the page.

Executive Summary

This analytical method, Monsanto Method AG-ME-1636-03, is designed for the quantitative determination of MON 102100 (tioxazafen) and its transformation product benzamidine in soil. The method is quantitative for the analytes at the stated LOQs of 0.005 mg/kg (ppm) for MON 102100 using ESI GC/MS/MS and 0.00125 mg/kg for benzamidine using ESI LC/MS/MS. The LOQs are less than the lowest toxicological level of concern in soil (chronic earthworm LOAEC = 95.3 mg a.i./kg; MRID 49304244) and sediment (marine amphipod NOAEC = 0.25 mg a.i./kg; MRID 49304228) for the analytes. The ILV was based on the previous version of the method, AG-ME-1636-02 versus AG-ME-1636-03, along with protocol amendments to extend the LOQ of benzamidine. None of the modifications of Method AG-ME-1636-02 to Method AG-ME-1636-03 were modifications of the extraction or analytical procedure. Method AG-ME-1636-02 was validated for both analytes in the third trial with only minor ILV modifications. The ECM contained pre-study validation data and in-study terrestrial field dissipation validation data; however, no samples were fortified at 10×LOQ for the MON 102100 validation. The ECM report did not provide adequate and accurate chromatograms to support the method. Also, the soil matrices of ECM pre-study validation were not characterized or described.

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						
MON 102100	49304239	49304240		Soil ¹	11/11/2014 (Method Dates 29/08/2014 & 10/06/2014) ²	Monsanto	GC/MS/MS	0.005 mg/kg
Benzamidine							LC/MS/MS	0.00125 mg/kg ³

- 1 For the ECM, the soil for the pre-study validation was not described or characterized. For the ECM in-study validations during a terrestrial field dissipation study (MRID 49304217), two sand soils from Georgia (3-5% clay, 0.59-0.72% organic matter), two clay loam soils from Nebraska (29-33% clay, 2.1-3.1% organic matter), clay loam soil (30% clay, 4.1% organic matter) and silty clay loam soil (34% clay, 3.8% organic matter) from Illinois, and two loamy sand soils from Manitoba, Canada (8-10% clay, 1.4-2.9% organic matter) were used. The soil matrices of the ILV were soil matrices were high sand soil (7% clay, 2.0% organic matter) and high clay soil (43% clay, 2.1% organic matter; sources not reported).
- 2 The method which was provided by the ECM was Monsanto Method AG-ME-1636-03 (Effective Date: August 29, 2014; Appendix I, pp. 30-56 of MRID 49304239). However, the ILV MRID 49304240 reported the name of method which was validated as Monsanto Method AG-ME-1636-02 [Effective Date: June 10, 2014; Appendix A, Appendix 1 (pp. 65-98) and Appendix E (pp. 121-150)]. None of the modifications of AG-ME-1636-02 to AG-ME-1636-03 were considered substantial - no modification of the extraction or analytical procedure.
- 3 Reported as the LOQ in ECM MRID 49304239 and the Protocol Amendments in ILV MRID 49304240, but not in Monsanto Methods AG-ME-1636-02 or AG-ME-1636-03 (where the LOQ was reported as 0.0013 mg/kg).

I. Principle of the Method

Subsamples (80 ± 5 mg) of frozen, homogenized, finely-milled soil were fortified, as necessary (40.0 μ L of the appropriate MON 102100 or benzamidine fortification solution in 65% acetonitrile in water solution), in 96-well format tubes (1.4-mL polypropylene tubes; pp. 12-13; Figure 1, p. 22; Appendix I, pp. 32-33, 37, 39-42 of MRID 49304239). 0.720 mL of the mixed stable-label internal standards solution in 65% acetonitrile in water solution was added to the samples. After a grinding ball was added to each tube, the samples were sonicated for *ca.* 3 minutes then shaken using a high-speed plate shaker (SPEX Geno/Grinder[®] 2010) set at 1200 cycles/minute for 30 minutes. After centrifugation ($\leq 10^{\circ}\text{C}$, 5 minutes at $6000 \times g$), the supernatants were transferred to separate, clean 96-well plates. For MON 102100 analysis in 96-well microplate with clear glass conical inserts, extracts were partitioned with toluene (1:1 volume with extract volume, 0.400 mL extract and toluene volume) via shaking using a high-speed plate shaker (SPEX Geno/Grinder[®] 2010) set at 1200 cycles/minute for 2 minutes. After centrifugation ($\leq 10^{\circ}\text{C}$, 5 minutes at $6000 \times g$), 0.100 mL of the toluene supernatants were transferred to separate, clean 96-well plates (same type as before or glass-lined polypropylene plate with cover and autosampler cap mat). The samples were taken for analysis by EI GC/MS/MS within the pre-determined storage time. For benzamidine analysis in Agilent 96-well plate (1 mL), extracts (0.050 mL) were diluted 15-fold with 0.700 mL of 95% acetonitrile in 10 mM ammonium formate (to adjust sample composition and pH for hydrophilic interaction chromatography). After mixing, the plate was covered with an autosampler cap mat, and the samples were analyzed by ESI LC/MS/MS.

The method noted that high-level samples with MON 102100 could be diluted with toluene so that the analyte response could be in the range of the calibration curve; however, dilution of samples for analysis of benzamidine was not approved due to failure to pass validation acceptance criteria (Appendix I, p. 42 of MRID 49304239).

Samples were analyzed for MON 102100 and the internal standard (phenyl- $^{13}\text{C}_6$) MON 102100 by ESI GC/MS/MS using an Agilent Model 7890A GC coupled to an Agilent Model 7000 Triple Quadrupole MS in electron impact MRM mode (p. 12; Appendix I, pp. 43-44 of MRID 49304239). The following GC conditions were used: DB-17 MS column (30 m x 0.25 mm, 0.25- μm film thickness), autosampler temperature 10°C , oven temperature program [90°C (initial), $40^{\circ}\text{C}/\text{min.}$ to 300°C , $10^{\circ}\text{C}/\text{min.}$ to 315°C , $0^{\circ}\text{C}/\text{min}$ for 5 minutes], detector temperature 230°C , and injection volume 0.5 μL . Two parent-daughter ion transitions were monitored (quantitative and confirmation, respectively): m/z 228 \rightarrow 119 and m/z 228 \rightarrow 111 for MON 102100, and m/z 234 \rightarrow 125 and m/z 234 \rightarrow 111 for (phenyl- $^{13}\text{C}_6$) MON 102100. Retention times for both MON 102100 and (phenyl- $^{13}\text{C}_6$) MON 102100 were observed at *ca.* 6.66-6.67 min. (Figure 4, p. 25) and *ca.* 7.03 min. (Appendix I, Appendix B, p. 50).

Samples were analyzed for benzamidine and the internal standard ($^{13}\text{C}_6$) benzamidine by ESI LC/MS/MS using a Shimadzu Prominence 20A system coupled to an AB Sciex API 5000/5500 Mass Spectrometer in positive ion MRM mode (p. 12; Appendix I, p. 45 of MRID 49304239). The instrumental conditions consisted of a Supelco Ascentis Express HILIC column (2.1 x 50 mm, 2.7- μm ; column temperature 40°C), a mobile phase gradient of (A) 50% methanol, 50 mM

ammonium formate and (B) 90% acetonitrile, 10 mM ammonium formate [percent A:B (v:v) at 0.0-1.8 min. 0:100, 1.8-4.1 min. 100:0, 4.2-8.0 min. 0:100] and MS/MS detection in electrospray ionization mode (temperature, 500°C), and injection volume 5 µL. Two parent-daughter ion transitions (quantitative and confirmation, respectively) were monitored: m/z 121.1 → 104.0 and m/z 121.1 → 77.0 for benzamidine, and m/z 127.1 → 110.0 and m/z 127.1 → 83.1 for ($^{13}\text{C}_6$) benzamidine. Retention times for both benzamidine and ($^{13}\text{C}_6$) benzamidine were observed at *ca.* 0.8 min. (Figure 5, p. 26) and *ca.* 1.46 min. (Appendix I, Appendix B, p. 49).

ILV

In the ILV, Method AG-ME-1636-02 (ME-1636) was performed exactly as written, except that the final aliquot in the MON 102100 preparation was transferred to an amber GC vial instead of 96-well plates (pp. 15-20, 25-26 of MRID 49304240). Samples were analyzed for MON 102100 and the internal standard (phenyl- $^{13}\text{C}_6$) MON 102100 by ESI GC/MS/MS using a GC Bruker 436 coupled to Bruker Scion MS in electron impact MRM mode. The same GC conditions were used, except that a Restek Rxi-17 sil MS column (30 m x 0.25 mm, 0.25-µm film thickness) was used. Samples were analyzed for benzamidine and the internal standard ($^{13}\text{C}_6$) benzamidine by ESI LC/MS/MS using an Agilent 1290 LC system coupled to an API 6500 Q-Trap MS/MS Detector in positive ion MRM mode. LC/MS/MS operating conditions were the same, including the column, except that the equilibration time at the end was adjusted [percent A:B (v:v) at 8.0-11.0 min. 0:100]. Also, additional fortification levels (4×LOQ and 40×LOQ) and lower calibration standards were added to the benzamidine validation. The same MRM transitions were monitored for all analytes and internal standards.

The ILV was based on the previous version of the method, AG-ME-1636-02 versus AG-ME-1636-03, along with protocol amendments to extend the LOQ of benzamidine (Appendix A, Appendix 1, pp. 65-98; Appendix E, pp. 121-150 of MRID 49304240). None of the modifications of Method AG-ME-1636-02 to Method AG-ME-1636-03 were considered substantial - no modification of the extraction or analytical procedure (see Reviewer's Comment #1).

LOQ/LOD

The LOQs in the ECM and ILV were reported as 0.0050 mg/kg (ppm) for MON 102100 and 0.00125 mg/kg (ppm) for benzamidine (pp. 10, 15-16 of MRID 49304239; pp. 15, 27 of MRID 49304240). The LODs for MON 102100 and benzamidine were 0.0022 mg/kg and 0.00027 mg/kg, respectively, in the ECM; the LODs were not reported in the ILV.

II. Recovery Findings

ECM (MRID 49304239: Method AG-ME-1636-03): Mean recoveries and relative standard deviations (RSDs) were within guidelines (mean 70-120%; RSD \leq 20%) for analysis of MON 102100 at fortification levels of 0.005 mg/kg (LOQ), 0.100 mg/kg (20 \times LOQ), and 0.9 mg/kg (180 \times LOQ) for soil in pre-study and in-study validations (p. 16; Tables 1-4, pp. 20-21; Appendix I, Appendix C, pp. 53-54 of MRID 49304239). No samples were prepared at 10 \times LOQ with MON 102100. Mean recoveries and relative standard deviations (RSDs) were within guidelines (mean 70-120%; RSD \leq 20%) for analysis of benzamidine at fortification levels of 0.0013 mg/kg (initial LOQ), 0.013 mg/kg (10 \times LOQ), and 0.24 mg/kg (*ca.* 185 \times LOQ) for soil in pre-study validations and 0.00125 mg/kg (final LOQ), 0.0125 mg/kg (10 \times LOQ), and 0.30 mg/kg (240 \times LOQ) for soil in in-study validations. Only data from the primary ion transition was reported; the confirmation ion were monitored for determination of selectivity of the method (Appendix I, Appendix C, p. 52 of MRID 49304239). Pre-Study Validation Results were not corrected for residues in the controls (pp. 13-14 of MRID 49304239; Appendix 1, p. 83 of MRID 49304240). In-Study Validation results were corrected when residues were quantified in the controls (Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217). The soil for the pre-study validation was not described or characterized; the eight soils used for the in-study validation were two sand, three clay loam, silty clay loam and two loamy sandy soils (p. 12, 18 of MRID 49304239; Appendix 1, pp. 68-75 of MRID 49304240; Tables 2-5, pp. 58-61; Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217). Soil samples for the in-study validation during the terrestrial field dissipation study were taken from the 0-3", 3-6" and 6-12" soil depths and fully characterized by Agvise Laboratories, Northwood, North Dakota. Two sand soils from Georgia (3-5% clay, 7-9% silt, 88% sand; 0.59-0.72% organic matter), two clay loam soils from Nebraska (29-33% clay, 41-45% silt, 26% sand; 2.1-3.1% organic matter), one clay loam soil (30% clay, 49% silt, 21% sand; 4.1% organic matter) and one silty clay loam soil (34% clay, 47% silt, 19% sand; 3.8% organic matter) from Illinois, and two loamy sand soils from Manitoba, Canada (8-10% clay, 7-9% silt, 81-85% sand; 1.4-2.9% organic matter) were used.

ILV (MRID 49304240: Method AG-ME-1636-02): Mean recoveries and relative standard deviations (RSDs) were within guidelines (mean 70-120%; RSD \leq 20%) for analysis in high sand and high clay soils of MON 102100 at fortification levels of 0.005 mg/kg (LOQ) and 0.05 mg/kg (10 \times LOQ) and benzamidine at fortification levels of 0.00125 mg/kg (LOQ), 0.005 mg/kg (4 \times LOQ), 0.0125 mg/kg (10 \times LOQ), and 0.05 mg/kg (40 \times LOQ; Tables 5-8, pp. 33-34). Only data from the primary ion transition was reported. Soil matrices were fully characterized by Agvise Laboratories, Northwood, North Dakota (USDA texture classification; purchased soils; source not reported; p. 14; Appendix C, pp. 105-106). High sand soil (7% clay, 4% silt, 89% sand; 2.0% organic matter) and high clay soil (43% clay, 28% silt, 29% sand; 2.1% organic matter) were used. Method AG-ME-1636-02 was validated for both analytes in the third trial (Appendix A, p. 97).

Table 2. Initial Validation Method Recoveries for MON 102100 and Its Transformation Product Benzamidine in Soils using Method AG-ME-1636-03¹

Analyte	Fortification Level (mg/kg)	Number of Tests	Recovery Range (%) ²	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
Pre-Study Validation – Uncharacterized Soil						
MON 102100	0.005 (LOQ)	6	84-94	91	3.7	4.1
	0.10	6	88-92	90	1.4	1.5
	0.90	6	92-94	93	0.5	0.5
Benzamidine	0.0013 (LOQ) ³	5	83-96	92	5.5	6.0
	0.013	6	91-98	96	2.9	3.1
	0.24	6	96-101	98	1.5	1.6
In-Study Validation – Four Soils ⁴ (summarized data from MRID 49304217)						
MON 102100	0.005 (LOQ)	156	-- ²	96	9.6	10.0
	0.10	188	--	97	4.5	4.6
	0.90	171	--	99	4.1	4.1
Benzamidine	0.00125 (LOQ) ³	170	--	91	8.8	9.6
	0.0125	171	--	97	4.4	4.6
	0.30	156	--	99	3.7	3.7

Data (results) were obtained from Tables 1-4, pp. 20-21; Appendix I, Appendix C, pp. 53-54 of MRID 49304239 and Appendix 1, Appendix C, p. 94 of MRID 49304240 (recovery ranges of pre-study validation). Pre-Study Validation Results were not corrected for residues in the controls (pp. 13-14 of MRID 49304239; Appendix 1, p. 83 of MRID 49304240). In-Study Validation results were corrected when residues were quantified in the controls (Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217).

1 Only the primary ion transition recoveries were reported.

2 Individual recovery data was not provided for any analysis (see Reviewer Comment #6).

3 0.0013 mg/kg was the LOQ determined in the pre-study validation; 0.00125 mg/kg was the lower LOQ determined during in-study method validation assessments (p. 16 of MRID 49304239; see Reviewer Comment #2).

4 Soil samples for the in-study validation during the terrestrial field dissipation study were taken from the 0-3", 3-6" and 6-12" soil depths and fully characterized by Agvise Laboratories, Northwood, North Dakota (Tables 2-5, pp. 58-61; Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217). Two sand soils from Georgia (3-5% clay, 7-9% silt, 88% sand; 0.59-0.72% organic matter), two clay loam soils from Nebraska (29-33% clay, 41-45% silt, 26% sand; 2.1-3.1% organic matter), clay loam soil (30% clay, 49% silt, 21% sand; 4.1% organic matter) and silty clay loam soil (34% clay, 47% silt, 19% sand; 3.8% organic matter) from Illinois, and two loamy sand soils from Manitoba, Canada (8-10% clay, 7-9% silt, 81-85% sand; 1.4-2.9% organic matter) were used.

Table 3. Independent Validation Method Recoveries for MON 102100 and Its Transformation Product Benzamidine in Soils using Method AG-ME-1636-02^{1,2}

Analyte	Fortification Level (mg/kg)	Number of Tests	Recovery Range (%)	Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
High Sand Soil						
MON 102100	0.005 (LOQ)	5	71-92	80	8	10
	0.05	5	104-106	104	1	1
Benzamidine	0.00125 (LOQ)	5	87-98	91	4	5
	0.005	5	90-103	96	6	6
	0.0125	5	82-96	92	6	6
	0.05	5	90-109	100	7	7
High Clay Soil						
MON 102100	0.005 (LOQ)	5	78-98	84	8	10
	0.05	5	100-109	105	3	3
benzamidine	0.00125 (LOQ)	5	82-113	94	13	14
	0.005	5	70-102	84	13	16
	0.0125	5	87-105	94	8	8
	0.05	5	103-126	110	9	8

Data (uncorrected recovery results, pp. 20-23 and Tables 1-4, pp. 29-32) were obtained from Tables 5-8, pp. 33-34 of MRID 49304240.

1 Soil matrices were fully characterized by Agvise Laboratories, Northwood, North Dakota (USDA texture classification; purchased soils; source not reported; p. 14; Appendix C, pp. 105-106). High sand soil (7% clay, 4% silt, 89% sand; 2.0% organic matter) and high clay soil (43% clay, 28% silt, 29% sand; 2.1% organic matter) were used.

2 Only the primary ion transition recoveries were reported.

III. Method Characteristics

In the ECM and ILV, the LOQs in the ECM and ILV were reported as 0.0050 mg/kg (ppm) for MON 102100 and 0.00125 mg/kg (ppm) for benzamidine (pp. 10, 15-16 of MRID 49304239; pp. 15, 27 of MRID 49304240). The LOQs were established based on the successful validation of results at the fortification levels (mean recovery, 70-120%; RSD \leq 20%). The LOD values were calculated based on the following equation using validation data:

$$\text{LOD} = t_{0.99} \times \text{STDEV (for N-1 degrees of freedom)}$$

Where, $t_{0.99}$ is the one-sided t-test value at the 99 % confidence interval, STDEV is the standard deviation of the fortified replicates at the lowest acceptable fortification level and N is the number of replicate. The LODs for MON 102100 and benzamidine were calculated as 0.0022 mg/kg and 0.00027 mg/kg, respectively, in the ECM; the LODs were not reported in the ILV.

Table 4. Method Characteristics¹

Method		MON 102100	Benzamidine
Limit of Quantitation (LOQ)		0.005 mg/kg	0.00125 mg/kg ²
Limit of Detection (LOD)		0.0022 mg/kg	0.00027 mg/kg
Linearity (Least squares calibration curve r and concentration range)	ECM ³	r ² = 0.99989147 0.0040-1.0 mg/kg	r ² = 0.9988-0.9996 0.0010-0.30 mg/kg
	ILV	r ² = 0.99927 (Q) 0.0040-1.0 mg/kg	r ² = 0.9995 (Q) 0.00025-0.30 mg/kg
Repeatable	ECM ⁴	Yes at LOQ, 20×LOQ and 180×LOQ. No samples were prepared at 10×LOQ.	No samples prepared at LOQ or 10×LOQ. Yes for samples prepared at LOQ, 10×LOQ and <i>ca.</i> 185×LOQ using LOQ of 0.0013 mg/kg.
			In-study
	ILV ⁵	Yes at LOQ and 10×LOQ.	
Reproducible		Yes	
Specific	ECM/ILV	Only quantitation ion results were reported for GC/MS/MS or LC/MS/MS analysis. ⁶	
	ECM ⁷	Matrix interferences appeared to be 25% of the LOQ based on peak height using raw data provided in MRID 49304217. ⁸ Reported that 2 of the 89 control samples had interferences of >30% of the LOQ. ⁹	Matrix interferences were 25% of the LOQ based on peak area for the representative soil (NE clay loam). Reported that 27 of 88 control samples had interferences of 30-45% of the LOQ. ⁹
		Chromatograms of 10×LOQ were not provided. Chromatograms of the confirmation ion were not provided. No chromatograms were provided for the pre-study validation.	
ILV	Matrix interferences were <10% of the LOQ for the quantitative and confirmatory ions.	Matrix interferences were <10% of the LOQ for the quantitative ion, but peaks were broad. Confirmatory ion signals were barely visible above the baseline.	

Data were obtained from pp. 10, 14-17; Tables 1-4, pp. 20-21; Figures 4-7, pp. 25-27 of MRID 49304239; Appendix 3, Appendix A, pp. 680-707; Appendix 3, Appendix C, pp. 1030-1068 of MRID 49304217; pp. 15, 24-25, 27; Tables 1-9, pp. 29-35; Figures 1-20, pp. 36-55; Appendix D, pp. 113-119 of MRID 49304240; and DER Attachment 2. Q = quantitation ion.

1 The ECM provided Monsanto Method AG-ME-1636-03 as the analytical method; the ILV provided Monsanto Method AG-ME-1636-02 as the analytical method. Method AG-ME-1636-03 was an updated version of Method AG-ME-1636-02. None of the modifications of Method AG-ME-1636-02 to Method AG-ME-1636-03 were considered substantial - no modification of the extraction or analytical procedure (see Reviewer's Comment #1)..

2 The LOQ for benzamidine in the pre-study validation was 0.0013 mg/kg (pp. 15-16; Tables 1-4, pp. 20-21 of MRID 49304239). The LOQ was "extended to 0.00125 mg/kg (ppm) during in-study method validation assessments, therefore the LOQ was determined to be 0.00125 mg/kg (ppm)" (p. 16).

3 The coefficients of determination for benzamidine were reviewer-calculated from reported r values of 0.994 (Figure 7, p. 27 of MRID 49304239) and 0.9998 (Appendix 3, Appendix C, p. 1057 of MRID 49304217; see DER Attachment 2).

- 4 For the ECM pre-study validation, the soil was not described or characterized. For the ECM in-study validations during a terrestrial field dissipation study (MRID 49304217), two sand soils from Georgia (3-5% clay, 0.59-0.72% organic matter), two clay loam soils from Nebraska (29-33% clay, 2.1-3.1% organic matter), clay loam soil (30% clay, 4.1% organic matter) and silty clay loam soil (34% clay, 3.8% organic matter) from Illinois, and two loamy sand soils from Manitoba, Canada (8-10% clay, 1.4-2.9% organic matter) were used.
- 5 The soil matrices of the ILV were soil matrices were high sand soil (7% clay, 2.0% organic matter) and high clay soil (43% clay, 2.1% organic matter; sources not reported).
- 6 A confirmation method was not used in the method. However, confirmatory methods are typically not necessary where GC/MS and LC/MS methods are used as the primary method(s) to generate study data.
- 7 Insufficient chromatograms were provided in the ECM: no reagent blanks and 10×LOQ chromatograms were included (Figures 4-5, pp. 25-26). No chromatograms were included for the pre-study validation or confirmation ions. Chromatograms for the in-study validation were for a single soil, and the MON 102100 LOQ chromatogram was misnamed (actually 20×LOQ chromatogram). So, no LOQ chromatogram was provided for MON 102100 in the ECM MRID 49304239. See Reviewer's Comment #4 for more details.
- 8 Based on the chromatograms for MB control 0-3" 150 DAA and QC low 0.005 ppm MB, 3-6" 150 DAA (Appendix 3, Appendix C, pp. 1031, 1034, 1048 of MRID 49304217).
- 9 Summary of specificity on p. 15 of ECM MRID 49304239.

IV. Method Deficiencies and Reviewer's Comments

1. The method which was provided by the ECM was Monsanto Method AG-ME-1636-03 (Effective Date: August 29, 2014; Appendix I, pp. 30-56 of MRID 49304239). However, the ILV MRID 49304240 reported the name of method which was validated as AG-ME-1636 (pp. 11, 26) and Monsanto Method AG-ME-1636-02 [Effective Date: June 10, 2014; Appendix A, Appendix 1 (pp. 65-98) and Appendix E (pp. 121-150)]. The noted differences for AG-ME-1636-03 compared to AG-ME-1636-02 were as follows: 1) ARS solution stability study results were added; 2) increase in the concentrations of the intermediate calibration solutions of benzamidine; 3) minor changes to the soil homogenization example conditions; 4) minor amounts of additional guidance were added to the extraction procedure; 5) the change of the acceptance criterion for mean accuracy from "within 70-120% of the corrected, back-calculated nominal value" to "within 70-120% of the nominal value" (Appendix E, p. 134 of MRID 49304240; Appendix I, p. 42 of MRID 49304239, respectively); 6) the removal of the injection carryover guidance; and 7) several changes to the Appendix C Validation Summary, including the elimination of the recovery ranges for the pre-study validation and the addition of the summary of the in-study validation results. None of the modifications of AG-ME-1636-02 to AG-ME-1636-03 were considered substantial; however, the ILV should have been provided with the most up-to-date version of the method in order to perform the independent validation.

The modifications to the method by the ILV were not considered substantial: MON 102100 equipment changes, HPLC gradient extension for benzamidine analysis, and additional calibration points and fortification levels for benzamidine (pp. 25-26 of MRID 49304240). Modifications involving benzamidine were instructed by the sponsor.

The in-study validation performed during the terrestrial field dissipation study (MRID 49304217) provided Monsanto Method AG-ME-1636-03 (Appendix 3, Appendix A, pp. 680-707) which had been updated with "additional within-study validation and stability data" (p. 680).

2. The determination of the LOQ and LOD were not based on scientifically acceptable procedures as defined in 40 CFR Part 136, Appendix B. The LOQs were established based on the successful validation of results at the fortification levels (mean recovery, 70-120%; RSD \leq 20%; pp. 10, 15-16 of MRID 49304239). The LOD values were calculated based on the following equation using validation data: $LOD = t_{0.99} \times STDEV$ (for N-1 degrees of freedom), where $t_{0.99}$ is the one-sided t-test value at the 99 % confidence interval, STDEV is the standard deviation of the fortified replicates at the lowest acceptable fortification level and N is the number of replicate.

Detection limits should not be based on the arbitrarily selected lowest concentration in the spiked samples. Additionally, the lowest toxicological level of concern in soil was not reported. A LOQ above toxicological levels of concern results in an unacceptable method classification.

The LOQ for benzamidine in the pre-study validation was 0.0013 mg/kg (pp. 15-16; Tables 1-4, pp. 20-21 of MRID 49304239). The ECM reported the LOQ for benzamidine as 0.00125 mg/kg and described that the LOQ was “extended to 0.00125 mg/kg (ppm) during in-study method validation assessments, therefore the LOQ was determined to be 0.00125 mg/kg (ppm)” (p. 16). The reviewer noted that the extension of the LOQ for benzamidine to 0.00125 mg/kg was not reported in the Validation Summary of the Analytical Method AG-ME-1636-03 included with the ECM MRID 49304239 (Appendix I, pp. 30-56) and the Validation Summary of the Analytical Method AG-ME-1636-02 included with the ILV MRID 49304240 (Appendix E, pp. 121-150); however, it was included in the Protocol Amendments of the ILV Study Protocol (Appendix A, Appendix 1, pp. 65-98 of MRID 49304240).

Insufficient information was provided to determine if the LOQ is less than 10% of the expected or actual peak concentration of the test compound in the field (MRID 49304240). Following single applications of MON 102119 suspension concentrate formula to seeds (seed plot) at 314-392 g a.i./A (0.28-0.35 lb a.i./A) and to in-furrow plots at 310 g a.i./ha (0.28 lb a.i./A), maximum average concentrations (dry wt. basis) detected in the 0-3” soil depth were 1.5869-2.7939 for MON 102100 and 0.0063-0.0117 for benzamidine for seed plot and 0.6818-1.0899 for MON 102100 and 0.0014-0.0018 for benzamidine for in-furrow plots (p. 35; Tables 12-19, pp. 69-84 of MRID 49304217).

3. In the ECM, no performance data were provided for 10×LOQ to validate the method for MON 102100, only the LOQ, and 20×LOQ. A validation sample set should consist of, at a minimum, a reagent blank, two unspiked matrix control samples, five matrix control samples spike at the LOQ, and five matrix control samples spiked at 10×LOQ for each analyte and matrix.
4. Insufficient chromatograms were provided in the ECM: no reagent blanks and 10×LOQ chromatograms were included. No chromatograms were included for the pre-study validation or any confirmation ions. Chromatograms for the in-study validation were for a single soil, and the MON 102100 LOQ chromatogram was misnamed (actually 20×LOQ chromatogram). So, no LOQ chromatogram was provided for MON 102100 in the ECM MRID 49304239.

In the ECM MRID 49304239, only four chromatograms were provided for each analyte: one control sample for the analyte, one control sample with the internal standard (IS), one LOQ for analyte and one LOQ for the IS (Figures 4-5, pp. 25-26). These chromatograms were taken from the original report, the terrestrial dissipation study MRID 49304217. In the MRID 49304217 (Appendix 3, Appendix C, pp. 1030-1068), a variety of chromatograms were provided, but only two or three control chromatograms and one LOQ chromatogram total per analyte for all eight soil matrices and no 10×LOQ chromatograms.

Using the data in MRID 49304217, the reviewer noted that the MON 102100 LOQ chromatograms in ECM MRID 49304239 (Figure 4B, p. 25) were misnamed and should have been titled “NE QC Mid”, which corresponded to the Nebraska clay loam soil

fortified at 0.100 ppm, 6-12", -1 DAA (20×LOQ; based on the information on Appendix 3, Appendix C, pp. 1030, 1040 of MRID 49304217). The only LOQ chromatogram which was provided for MON 102100 in MRID 49304217 was QC low 0.005 ppm MB, 3-6", 150 DAA (Manitoba loamy sand soil, Appendix 3, Appendix C, pp. 1031, 1048 of MRID 49304217). The only MB control which was provided in MRID 49304217 was MB control 0-3" 150 DAA (Appendix 3, Appendix C, pp. 1030, 1040) – this was the representative control provided in ECM MRID 49304239. Regarding the benzamidine chromatograms, the reviewer noted that they were NE control 6-12" -1 DAA and QC low 0.00125 ppm NE 6-12", -1 DAA (Appendix 3, Appendix C, pp. 1051, 1064), so these two chromatograms were represented properly in ECM MRID 49304239.

5. In the ECM, In-Study Validation results were corrected when residues were quantified in the controls (Pre-Study Validation Results were not corrected; Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217; pp. 13-14 of MRID 49304239; Appendix 1, p. 83 of MRID 49304240). Recoveries were not corrected in the ILV (pp. 20-23 and Tables 1-4, pp. 29-32 of MRID 49304240).
6. The ECM MRID 49304239 did not provide raw data or recovery ranges for any of the validation study.

Data for recovery results for the in-study method validation portion of the ECM (Tables 3-4, p. 21 of MRID 49304239) were obtained from the 2014 terrestrial dissipation study reported in MRID 49304217 (see References). The raw data for the summarized in-study data reported in Tables 3-4 of ECM MRID 49304239 was found in Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217. In MRID 49304217, the statistics were calculated for each fortification level and soil, but separated by soil depth; individual recoveries were not reported. The recovery ranges for in-study results were not provided in Tables 3-4 of MRID 49304239. The reviewer attempted to go back to the raw data in MRID 49304217 in order to report these recovery ranges; however, the reviewer could not determine which raw data soil depth sets corresponded to the summarized data in the ECM MRID 49304239, e.g. for MON 102100 LOQ 0.005 ppm, number of tests = 156, but the sum of the 0-3" 0.005 ppm MON 102100 tests for all four soils = 24+29+36+66 = 155; and for benzamidine LOQ 0.00125 ppm, number of tests = 170, but the sum of the 0-3" 0.00125 ppm benzamidine tests for all four soils = 56+40+32+20 = 148 (Tables 3-4, p. 21 of MRID 49304239; Appendix 3, Tables 9-12, pp. 671-674 of MRID 49304217). Additionally, two additional fortifications for MON 102100 were validated in the in-study validation: 2.5 ppm (500×LOQ) and 4 ppm (800×LOQ); these results were not reported in ECM MRID 49304239.

Data for recovery results for the pre-study method validation portion of the ECM (Tables 1-2, p. 20 of MRID 49304239) were obtained from the ILV MRID 49304240 in Appendix A, Appendix 1 (pp. 65-98) and Appendix E (pp. 121-150) which reported Monsanto Method AG-ME-1636-02. The recovery ranges for pre-study results were not provided in Tables 1-2, p. 20 of MRID 49304239.

7. In the ECM, the soil matrices were not characterized for the pre-study validation (p. 12 of MRID 49304239). The study author reported that “pre-study data was archived with method validation for ME-1636” (p. 18). The original method ME-1636 was not included in the MRIDs submitted for the method validation or the terrestrial field dissipation (MRIDs 49304239, 49304240 & 49304217).
8. In the ECM, only representative calibration curves were provided (Figures 6-7, p. 27 of MRID 49304239). The raw data for these calibration curves were provided in the 2014 terrestrial dissipation study reported in MRID 49304217 (Appendix 3, Appendix C, pp. 1030-1068 of MRID 49304217). The calibration curves were shown for only one soil from in-study validation per analyte (quantitative ion only); no calibration curves were shown for pre-study validation.

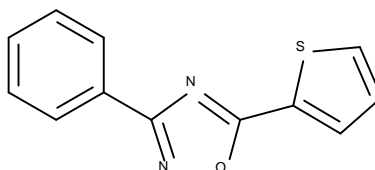
In the ILV, only representative calibration curves were provided, one per analyte (quantitative ion only; Figures 1-20, pp. 36-55 of MRID 49304240).
9. A confirmation method was not used in the method. However, confirmatory methods are typically not necessary where GC/MS and LC/MS methods are used as the primary method(s) to generate study data.
10. In the ILV, the study author reported that communications with the sponsor were necessary (p. 26; Appendix B, pp. 100-103 of MRID 49304240). These communications involved the LC/MS/MS system and failed trials of benzamidine.
11. The reviewer noted the possibility that an outlier was discarded for benzamidine fortified at 0.0013 mg/kg in the results of the pre-study validation (Appendix 1, Appendix C, p. 94 of MRID 49304240). The method directed that “a maximum of 1 outlier...may be discarded at each fortification level”, and the number of tests was 5 for the LOQ, while they were 6 for the other two fortification levels.
12. The method AG-ME-1636-03 noted that, for MON 102100 analysis, sample may be gently evaporated to *ca.* 0.150-0.200 mL in order to remove part or all of the acetonitrile solvent prior to the addition of toluene (Appendix I, p. 41 of MRID 49304239). Pre-study method validations with partial and complete acetonitrile evaporation prior to reconstitution in toluene yielded acceptable results; however, the in-study validation and ILV validation excluding the evaporation step due extraction (Tables 1-4, pp. 20-21 of MRID 49304239; pp. 16-17 of MRID 49304240).
13. In the ECM Method AG-ME-1636-03, the method reported that ionization effects were not significant (Appendix I, Appendix C, p. 55 of MRID 49304239).
14. In the ECM Method AG-ME-1636-03, the stability of the processed samples (post-extraction) was determined to be 5 days for MON 102100 and 8 days for benzamidine at *ca.* 4°C (Appendix I, Appendix C, p. 55 of MRID 49304239).
15. The time requirement for completion of the method was not reported in the ECM or ILV.

V. References

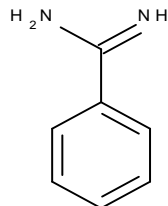
- EPA MRID No. 49304217. Jacobson, B., M.G. Mueth, L.S. Riter. 2014. Terrestrial Field Dissipation of MON 102100 Applied as a Seed Treatment Under Field Conditions at Four Regional North American Locations. Monsanto Report No.: MSL0025797. Monsanto Company Study No.: WBE-2013-0119. Waterborne Study No.: 666.06. EPL BAS Study No.: 115G624. Report prepared by Waterborne Environmental, Inc. (WEI), Leesburg, Virginia; Monsanto Company, St. Louis, Missouri; EPL Bio Analytical Services (EPL BAS), Niantic, Illinois; and Agvise Laboratories, Northwood, North Dakota; and sponsored and submitted by Monsanto Company, St. Louis, Missouri; 1162 pages. Final report issued November 21, 2014.
- 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**MON 102100 (Tioxazafen)****IUPAC Name:** 3-Phenyl-5-thiophen-2-yl-[1,2,4]oxadiazole

3-Phenyl-5-thiophen-2-yl-1,2,4-oxadiazole

CAS Name: 3-Phenyl-5-(2-thienyl)-1,2,4-oxadiazole**CAS Number:** 330459-31-9**SMILES String:** c1ccc(cc1)c2nc(on2)c3cccs3**Benzamidine****IUPAC Name:** Benzamidine

Benzenecarboximidamide

CAS Name: Not reported**CAS Number:** 618-39-3**SMILES String:** c1ccccc1C(=N)N**Attachment 1: Calculations**

Calcs