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Title

Independent Laboratory Validation of Bayer Method PI-003-S16-01: An Analytical Method for the Determination of Residues of Pyrimethanil (AE B100309) and its Metabolite AE F132593 in Soil and Water Using LC/MS/MS

- Final Report -

Test Item

Pyrimethanil and its Metabolite AE F132593

Data Requirement

US EPA OCSPP 850.6100, 860.1340

GENERAL INFORMATION

Contributors

The following people contributed to this report in the capacities indicated:

Name	Title
Fenn Li	Team Leader, Study Director
Sidney Hill	Technician
Allison Ricko	Scientist
Thomas Gould	Sponsor Representative/Study Monitor (Bayer CropScience)

Study Dates

Study initiation date: 16 Nov 2016
Experimental start date: 16 Nov 2016
Experimental termination date: 04 Dec 2016
Study completion date: See title page

Protocol Amendments and Deviations

None

Deviations from the Guidelines

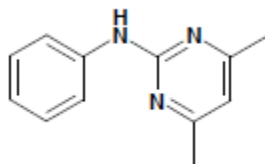
None

Retention of Samples

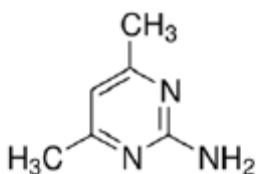
None necessary

Analytical Reference Standards

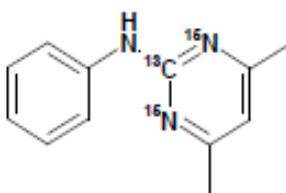
Standard name: Pyrimethanil (AE B100309)
Standard number: K-1342
CAS name: 4,6-Dimethyl-*N*-phenyl-2-pyrimidinamine
CAS number: 53112-28-0
Molecular formula: C₁₂H₁₃N₃
Molecular weight: 199.25 g/mol
GLP purity: 99.9%
Expiration date: 30 Jul 2018
Storage conditions: Frozen
Molecular structure:



Standard name: AE F132593
Standard number: K-2223
CAS name: 4-Dimethyl-2-pyrimidinamine
CAS number: 767-15-7
Molecular formula: C₆H₉N₃
Molecular weight: 123.16 g/mol
GLP purity: 97.5%
Expiration date: 19 Sep 2022
Storage conditions: Frozen
Molecular structure:



Standard name: Pyrimethanil-¹³C, ¹⁵N₂ (IS)
Standard number: K-1915
CAS name: Not available
CAS number: Not available
Molecular formula: C₁₂H₁₃N₃
Molecular weight: 202.23 g/mol
GLP purity: 99.8%
Expiration date: 23 Apr 2020
Storage conditions: Frozen
Molecular structure:



Other

Upon completion of the study, a copy of the protocol and the final report will be archived at CPS. The original protocol, final report, raw data, correspondence, and other documentation will be transferred to the Bayer CropScience Archives, Bayer CropScience, 2 T.W. Alexander Drive, RTP, NC 27709.

2.0 INTRODUCTION

The objective of this study was to validate Bayer Method PI-003-S16-01: “An Analytical Method for the Determination of Residues of Pyrimethanil (AE B100309) and its Metabolite AE F132593 in Soil and Water Using LC/MS/MS” [1].

This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSP 850.6100 [2] and 860.1340 [3]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [4].

3.0 MATERIALS AND METHODS

3.1 Test Substance and Internal Standard

Test Substance

Standard name: Pyrimethanil (AE B100309)
Standard no.: K-1342
CAS name: 4,6-Dimethyl-*N*-phenyl-2-pyrimidinamine
CAS no.: 53112-28-0
GLP purity: 99.9%
Expiration date: 30 Jul 2018
Storage conditions: Freezer

Standard name: AE F132593
Standard no.: K-2223
CAS name: 4-Dimethyl-2-pyrimidinamine
CAS no.: 767-15-7
GLP purity: 97.5%
Expiration date: 19 Sep 2022
Storage conditions: Freezer

Internal Standard (IS)

Standard name: Pyrimethanil-¹³C, ¹⁵N₂ (IS)
Standard no.: K-1915
CAS name: Unavailable
CAS no.: Unavailable
GLP purity: 99.8%
Expiration date: 23 Apr 2020
Storage conditions: Freezer

3.2 Test System

The test systems used for the validation were a water sample (CPS ID GS-16-179-2) and a soil sample (CPS ID GS-16-179-1) provided by Bayer CropScience. The water and soil samples were held in a refrigerator at 2 to 8°C (CPS Equipment ID REF.1.14) until needed for analysis.

3.3 Equipment and Reagents

The equipment and reagents used for the independent laboratory validation (ILV) were as outlined in Bayer Method P1-003-S16-01 (Appendix 4; Section 3.0: Apparatus and Section 4.0: Reagents and Consumables). Identical or equivalent apparatuses and materials were used.

3.3.1 Equipment and Apparatus

- Volumetric flasks, glass class A (assorted volumes)
- Top-loading balance (A&D Company)
- Electronic pipettors, various volumes (Eppendorf)
- Manual pipettors, various volumes (VWR, Brand)
- Pasteur pipettes (VWR part no. 414004-007)
- Scintillation vials, 20 mL (VWR part no. 56941-386)
- HPLC vials and caps, 2 mL (VWR part no. 46610-722)
- Rotary evaporator, RC 600 (KNF)
- Vortex mixer (VWR)
- Soxhlet extractors and condensers
- Ultrasonic cleaner (Branson)
- Boiling flasks, 250 mL
- Heating block (Electrothermal)
- Extraction thimbles, cellulose 25 mm × 80 mm (Whatman)
- Kinetex[®], 2.6 µm XB-C18, 100 × 2.1 mm (Phenomenex part no. 00D-4496-AN)
- LC-MS/MS—Shimadzu Nexera X2 Modular UHPLC system with a PAL autosampler (CTC Analytics) coupled to a Sciex[®] Triple Quad™ 6500 tandem mass spectrometer with an electrospray ionization interface and Analyst[®] 1.6.2 data collection software
- Various general laboratory glassware and utensils

3.3.2 Reagents

- Water (Milli-Q[®])
- Acetonitrile (ACN; BDH, HPLC grade, Lot# 16E261212)
- Methanol (MeOH; BDH, HPLC grade, Lot# 16D054014)
- Ammonium formate (NH₄HCO₂; Sigma-Aldrich, Lot# MKBS1777V)
- Formic acid 99% (Amresco, Lot# 2515C292)
- ACN:water (1:9, v/v) prepared by adding 100 mL of water to 900 mL of ACN.

- MeOH:water (1:9, v/v) containing 10 mM ammonium formate and 120 µL/L formic acid; prepared by adding 0.63 g of ammonium formate and 120 µL of formic acid to 900 mL of water and mixing, then adding 100 mL of MeOH.
- MeOH:water (9:1, v/v) containing 10 mM ammonium formate and 120 µL/L formic acid; prepared by adding 0.63 g of ammonium formate and 120 µL of formic acid to 100 mL of water and mixing, then adding 900 mL of MeOH.

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection sensitivity, and linearity of instrument responses to a range of analyte concentrations were determined.

3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

Sample validation sets

Each analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with test substances at the LOQ, and five untreated controls fortified with test substances at 10× LOQ.

Data are summarized in Table 1–Table 2 and Table 3–Table 4 for water and soil samples, respectively. Residue data sheets are included in Appendix 1.

Calibration standard solutions (0.150 to 50.0 ng/mL in water analysis and 1.50 to 1500 ng/mL in soil analysis for test substances and 50.0 ng/mL for IS) and a solvent blank were also included in each sample analysis batch.

Fortification

The water control LOQ and 10× LOQ samples were fortified with 100 µL of the fortification standard solutions, FS-0292-04 (50.0 ng/mL) for LOQ and FS-0292-03 (500 ng/mL) for 10× LOQ.

The soil control LOQ and 10× LOQ samples were fortified with 100 µL of the fortification standard solutions, FS-0292-03 (500 ng/mL) for LOQ and FS-0292-02 (5000 ng/mL) for 10× LOQ.

Extraction and workup for water samples

The following extraction steps were followed for each water sample.

1. Weigh 10 ± 0.1 g of water into a 20-mL glass scintillation vial.
2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution (see Section 3.4.4).
3. Add 100 µL of the 5.00 µg/mL IS solution IS-0292-01 to each sample and vortex well.
4. Transfer a portion of sample to an HPLC vial for analysis by LC-MS/MS.

Extraction and workup for soil samples

The following extraction steps were followed for each soil sample.

1. Weigh 10.0 ± 0.1 g of soil into a Soxhlet extraction thimble.
2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution (see Section 3.4.4).
3. Lightly plug the top of the Soxhlet extraction thimble with glass wool.
4. Transfer 125 mL of extraction solution (9:1 ACN:water) into a 250-mL boiling flask.
5. Place the boiling flask on a heating block, followed by a Soxhlet extractor containing the extraction thimble with the sample, followed by a condenser.
6. Allow the apparatus to reflux and cycle for 6 hours.
7. Allow the sample to cool down.
8. Rinse the condenser with 15.0 mL of ACN and collect the rinse in the soxhlet extractor.
9. Disassemble the Soxhlet apparatus and decant any residual solvent from the apparatus into the 250-mL boiling flask.
10. Add 100 μ L of IS-0292-01 (5.00 μ g/mL) into each sample and swirl to mix.
11. Pipette 14.0 mL of sample into a 250-mL boiling flask.
12. Evaporate the sample to near dryness on a rotary evaporator set at 45°C.
13. Add 1.00 mL of water to the boiling flask and sonicate.
14. Transfer sample to an HPLC vial for analysis by LC-MS/MS.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described in Bayer Method PI-003-S16-01. The mass spectrometer, Sciex[®] Triple Quad™ 6500 with an electrospray ionization interface, was optimized for the best sensitivities for the analytes.

3.4.4 Fortification and Calibration Standard Solutions Preparation

Fortification and calibration standard solutions were prepared following the methods below.

Primary stock standard solutions of pyrimethanil and AE F132593

Prepare individual stock solutions of approximately 2 mg/mL of pyrimethanil and AE F132593. Prepare the primary stock solution for each reference standard separately by pipetting 5.00 mL ACN into each pre-weighed standard (approximately 0.01 g) vial. Sonicate the vial until all material is dissolved completely.

Mixed standard (FS-0292-01 50.0 μ g/mL) of pyrimethanil and AE F132593

Pipette an appropriate volume of each of the primary stock solutions and dilute to 25.0 mL with ACN.

Mixed fortification standard (FS-0292-02 5.00 μ g/mL) of pyrimethanil and AE F132593

Transfer 2.50 mL of the 50.0 μ g/mL mixed standard solution into a 25-mL volumetric flask. Dilute to volume with ACN. Mix well.

Mixed fortification standard (FS-0292-03 0.500 µg/mL) of pyrimethanil and AE F132593

Transfer 2.50 mL of the 5.00 µg/mL mixed standard solution into a 25-mL volumetric flask. Dilute to volume with ACN. Mix well.

Mixed fortification standard (15CPS40-FS4 0.0500 µg/mL) of pyrimethanil and AE F132593

Transfer 2.50 mL of the 0.500 µg/mL mixed standard solution into a 25-mL volumetric flask. Dilute to volume with ACN. Mix well.

Primary stock solution of pyrimethanil IS

Prepare IS stock solutions of approximately 1 mg/mL of pyrimethanil IS by pipetting 5.00 mL ACN into the pre-weighed standard (approximately 0.005 g) vial. Sonicate the vial until all material is dissolved completely.

Pyrimethanil IS solution (IS-0292-01 5.00 µg/mL)

Pipette an appropriate volume of the primary stock IS into a 25-mL volumetric flask. Dilute to 10 mL with ACN.

Calibration standard solutions

Prepare working calibration solutions consisting of 0.150, 0.500, 1.50, 5.00, 15.0, 50.0, 150, 500, and 1500 ppb of pyrimethanil and AE F132593 by diluting to 25.0 mL with water. Before bringing the calibration solutions to volume, pipette 250 µL of the 5.00 µg/mL IS solution to each of the calibration solutions.

Concentration of Standard Solution Used for Dilution (µg/mL)	Concentration of IS Solution Used for Dilution (µg/mL)	Aliquot Native Mix Taken (mL)	Aliquot IS Taken (mL)	Concentration of Calibration Solution (ppb)	Concentration of IS (ppb)
0.0500	5.00	0.0750	0.250	0.150	50.0
0.0500	5.00	0.250	0.250	0.500	50.0
0.500	5.00	0.0750	0.250	1.50	50.0
0.500	5.00	0.250	0.250	5.00	50.0
5.00	5.00	0.0750	0.250	15.0	50.0
5.00	5.00	0.250	0.250	50.0	50.0
50.0	5.00	0.0750	0.250	150	50.0
50.0	5.00	0.250	0.250	500	50.0
50.0	5.00	0.750	0.250	1500	50.0

The primary standard solutions were stored in a freezer, and all other standard solutions were stored in a refrigerator when not in use.

3.5 LC-MS/MS Instrumentation

Shimadzu Nexera X2 Modular UHPLC system with a PAL autosampler (CTC Analytics)

Sciex[®] Triple Quad™6500 LC-MS/MS

Software: Sciex[®] Analyst[®] 1.6.2

HPLC column: Phenomenex Kinetex[®], 2.6 μm XB-C18, 100 × 2.1 mm (part no. 00D-4496-AN)

3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst[®] software version 1.6.2. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analytes of interest. The overall purpose of the external calibration curve was to display acceptable linearity ($r^2 \geq 0.99$) of the assigned calibration range. The recoveries of the analyte from the fortified samples were calculated by multi-point calibration.

Recovery results were computed for each sample. The equation used for quantification is presented in Appendix 2. A statistical treatment of the data includes the calculation of means, standard deviations (SD), and RSDs as percentages. All statistics were calculated using Microsoft[®] Excel[®] 2010.

Table 5 HPLC System Operating Parameters for Bayer Method PI-003-S16-01

HPLC System: Shimadzu Nexera X2 Modular UHPLC system
Autosampler: CTC Analytics PAL system
Software: Sciex[®] Analyst[®] 1.6.2

LC Conditions

Analytical Column: Phenomenex Kinetex[®] XB-C18, 2.6 μm , 100 \times 2.1 mm
Mobile Phase: (A): 9:1 (v/v) water:methanol containing 10 mM ammonium formate and 120 $\mu\text{L/L}$ formic acid
(B): 1:9 (v/v) water:methanol containing 10 mM ammonium formate and 120 $\mu\text{L/L}$ formic acid

Column Temperature: Ambient

Injection Volume: 10.0 μL for water and 4 μL for soil analysis

Injection Speed: 5.00 $\mu\text{L/second}$

Run Time: 5.0 minutes

Gradient:

Time (min.)	A (%)	B (%)	Flow ($\mu\text{L/min.}$)
0.00	95	5	250
0.10	95	5	250
1.00	5	95	250
2.50	5	95	250
2.51	95	5	250
5.00	95	5	250

Table 6 MS/MS Operating Parameters for Bayer Method PI-003-S16-01

Tandem Mass Spectrometry System, Sciex® Triple Quad™ 6500
 Software: Sciex® Analyst® 1.6.2

The following parameters were used for operation of the mass spectrometer:

Parameter	Setting
Ion Source:	Turbo Spray (electrospray ionization)
Scan Type:	MRM
Polarity:	Positive
Collision Gas (CAD):	9.00
Curtain Gas (CUR):	25.0
Ion Source Gas 1 (GS1):	50.0
Ion Source Gas 2 (GS2):	50.0
Ion Spray Voltage (IS):	5500
Temperature (TEM):	400
Interface Heater (IHE):	ON

Analyte Name	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP	CE	EP	CXP
Pyrimethanil—Primary	200	107	100	111	49	10	12
Pyrimethanil—Confirmatory	200	76.9	100	111	55	10	10
Pyrimethanil IS	203	110	100	91	33	10	14
AE F132593—Primary	124	107	100	91	27	10	12
AE F132593—Confirmatory	124	66.9	100	91	37	10	10

Appendix 2 Calculations

Residue concentrations were determined using calibration curves which were generated after each analysis using Sciex[®] Analyst[®] software (version 1.6.2) with linear regression and 1/x weighting. The results were exported to Microsoft[®] Excel[®] 2010 and further processed. Data summary documents were prepared with Microsoft[®] Excel[®] 2010.

The standards were fit to the linear equation:

$$Y = MX + B$$

where: X is the concentration in ppb

M is the slope

B is the intercept

Y is the peak area ratio (analyte/IS)

After regression coefficients were calculated, the residue found in ppb was determined using the following equation:

$$\text{Residue found in water (ppb)} = \frac{(Y - B)}{M}$$

$$\text{Residue found in soil (ppb)} = \frac{(Y - B) \times FV}{M \times (AF \times Wt)}$$

where: FV = final extract volume, 1.00 mL

AF = aliquot factor, 0.100

Wt = sample weight, g

Samples were fortified prior to extraction. Theoretical ppb in fortified sample can be calculated as follows:

$$\text{Theoretical ppb in fortified sample (ppb)} = \frac{C_{sp} \times V_{sp}}{Wt}$$

where: C_{sp} = spiking solution concentration

V_{sp} = spiking solution volume (0.100 mL)

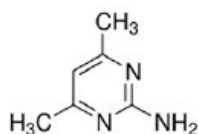
Wt = initial sample weight, g

Recoveries were calculated using the following equation:

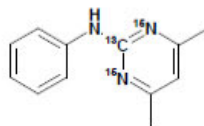
$$\text{Recovery (\%)} = \frac{\text{ppb found in fortified sample}}{\text{Theoretical ppb in fortified sample}} \times 100$$

Bayer CropScience Study Number: RAPIN005
CPS Study Number: 16-CPS-051

Standard name: AEF132593
Standard number: K-2223
CAS name: 4,6-Dimethyl-2-pyrimidinamine
CAS number: 767-15-7
Molecular formula: C₆H₈N₂
Molecular weight: 123.16 g/mol
GLP purity: 97.5%
Expiration date: 19 Sep 2022
Storage conditions: Frozen
Molecular structure:



Standard name: Pyrimethanil-¹³C,¹⁵N₂ IS
Standard number: K-1915
CAS name: Not available
CAS number: Not available
Molecular formula: C₁₂H₁₃N₃
Molecular weight: 202.23 g/mol
GLP purity: 99.8%
Expiration date: 23 Apr 2020
Storage conditions: Frozen
Molecular structure:



- 3.1.1 The reference standards will be supplied by Bayer CropScience. Methods of synthesis, fabrication, and/or derivation of the reference standards are maintained by Bayer CropScience.
- 3.1.2 Characterization data of this material and the retain will be archived at Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, North Carolina, 27709.
- 3.1.3 A record of storage conditions, weights, and dilutions of the reference standards will be maintained and checked by CPS quality control unit.