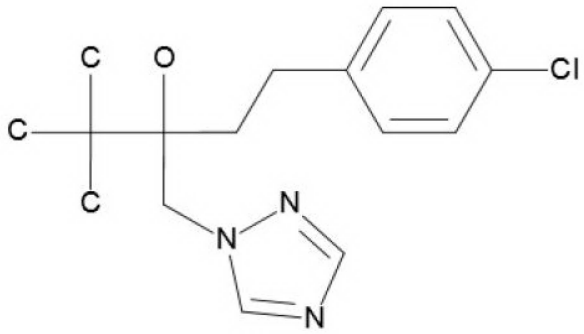
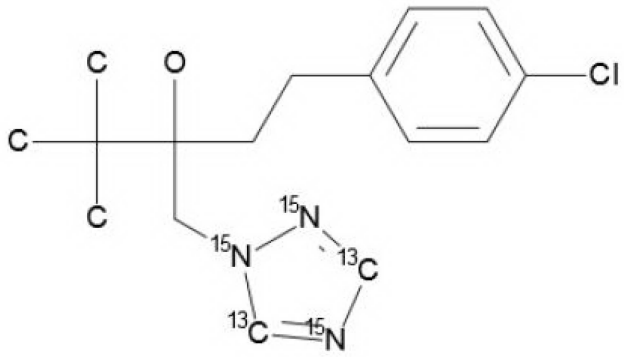
Analytical Reference Standards

Standard name:	Tebuconazole
Client standard number:	K-2213
CPS ID:	17-CPS-Mar23-01
CAS name:	α-[2-(4-Chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol
CAS number:	107534-96-3
Molecular formula:	$C_{16}H_{22}ClN_3O$
Molecular weight:	308 g/mol
GLP purity:	96.8%
Expiration date:	26 Apr 2023
Storage conditions:	Refrigerator
Structure:	



Standard name:	Tebuconazole-$^{13}C_2$,$^{15}N_3$
Client standard number:	K-2075
CPS ID:	17-CPS-Mar23-02
CAS number:	none
Molecular formula:	$^{13}C_2C_{14}H_{22}Cl^{15}N_3O$
Molecular weight:	311 g/mol
GLP purity:	100.0%
Expiration date:	13 Aug 2023
Storage conditions:	Freezer
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, Research Triangle Park, North Carolina, 27709.

1.0 EXECUTIVE SUMMARY

Bayer Method HW-005-W17-01 entitled, "An Analytical Method for the Determination of Residues of Tebuconazole in Drinking and Surface Water Using LC/MS/MS" [1], was validated successfully on the second trial. This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSPP 850.6100 [2] and OPPTS 860.1340 [3]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [4].

The method was successfully validated in the first trial for tebuconazole in water at the limit of quantitation (LOQ) and 10× LOQ concentration levels (0.050 and 0.500 ppb). However, due to contamination of the reagent blank and untreated controls, a second trial was conducted, and no contamination was observed.

The method was performed as written with one modification. The Mass Spectrometer TEM parameter was set to 450°C for increased sensitivity and reduced cone contamination. It took one person approximately 1 hour to complete the preparation of one set of 13 samples (one reagent blank, two unfortified matrix control samples, and 10 fortified samples). Time of analysis was approximately 3 hours. Complete analysis of one set, including sample preparation, analysis, and data processing, took approximately 1 day.

2.0 INTRODUCTION

The objective of this study was to validate Bayer Method HW-005-W17-01, "An Analytical Method for the Determination of Residues of Tebuconazole in Drinking and Surface Water Using LC/MS/MS" [1].

This study was designed to fulfill the requirements of the US EPA Test Guidelines OCSPP 850.6100 [2] and OPPTS 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:	Tebuconazole
Standard no.:	K-2213
CAS name:	α-[2-(4-Chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol
CAS no.:	107534-96-3
GLP purity:	96.8%
Expiration date:	26 Apr 2023
Storage conditions:	Refrigerator

Internal Standard

Standard name:	Tebuconazole-$^{13}C_2$,$^{15}N_3$
Standard no.:	K-2075
CAS name:	α-[2-(4-Chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1*H*-1,2,4-triazole-1,2,4-$^{15}N_3$-1-ethanol
CAS no.:	none
GLP purity:	100.0%
Expiration date:	13 Aug 2023
Storage conditions:	Freezer

3.2 Test System

The test system used for the validation was control water sample #031115-W (CPS ID GS-17-28-1) provided by Bayer CropScience. The water sample was held in a refrigerator at 2 to 8°C 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 HW-005-W17-01 (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)
- Electronic pipettors, various volumes (Eppendorf)
- Manual pipettors, various volumes (VWR, Brand)
- HPLC vials and caps, 2 mL (VWR)
- Vortex mixer (VWR)
- LC-MS/MS—Agilent 1200 Series Modular HPLC system coupled to a Sciex® Triple Quad™ 4000 tandem mass spectrometer with an electrospray ionization interface and Analyst® 1.6.2 data collection software
- Luna®, 2.5 µm C18(2)-HST, 50 × 2 mm (Phenomenex® part no. 00B-4446-B0)
- Various general laboratory glassware and utensils
- Vortex mixer

3.3.2 Reagents and Solutions

- Milli-Q® Water, Millipore Direct-Q5
- Acetonitrile (ACN; BDH, HPLC grade)
- Formic acid, 98% (Fluka®)
- 0.01% formic acid in water; prepared by adding 0.0500 mL formic acid to 500 mL water
- 1.0% formic acid in water; prepared by adding 1.00 mL formic acid to 100 mL water
- 0.1% formic acid in water: prepared by adding 1.00 mL formic acid to 1000 mL water
- 0.1% formic acid in acetonitrile: prepared by adding 1.00 mL formic acid to 1000 mL acetonitrile

3.4 Experimental Design

3.4.1 Establishment of the Method

The mass spectrometer, Sciex® Triple Quad™ 4000 with an electrospray ionization interface, was optimized for the best sensitivities for the analytes. 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
The analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with the test substance at the LOQ, and five untreated controls fortified with the test substance at 10× LOQ.

Data are summarized in Table 1 and Table 2. Residue data sheets are included in Appendix 1.

Calibration standard solutions (0.0250 to 1.00 ppb and 0.100 ppb for IS) and a solvent blank were also included in each sample analysis batch.

Fortification
Samples were fortified at the LOQ or 10× LOQ by adding 50 µL of the 0.0100 µg/mL or the 0.100 µg/mL secondary standard solution, respectively, to 10 mL of the control water sample (Section 3.2).

Extraction and workup for water samples
The following extraction steps were followed for each water sample.

1. Measure 10-mL of sample into a 20-mL scintillation vial.
2. Fortify the recovery samples at the desired fortification level with the appropriate standard solution (see Section 3.4.4).
3. Add 0.200 mL of the 0.005 µg/mL IS solution to each sample.
4. Add 0.1 mL of 1% formic acid to each sample, cap, and mix.
5. Transfer an aliquot of the 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 HW-005-W17-01. The validation was considered acceptable if the mean recovery of each fortification level at or above the LOQ was between 70% and 120% and if the RSD for replicate measurements is ≤20%. The control matrix was essentially free of any interference at the retention time of the analyte and internal standard.

3.4.4 Fortification and Calibration Standard Solutions Preparation

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

Primary stock reference (native) standard solution of tebuconazole
Prepare the primary stock solution for the tebuconazole reference standard by pipetting 5.00 mL acetonitrile into the pre-weighed (approximately 0.010 g) standard vial. Mix well.

Secondary standard solution (10.0 µg/mL) of tebuconazole
Pipette an appropriate volume of the primary stock solution into a 100-mL volumetric flask. Dilute with acetonitrile to get a concentration of 10.0 µg/mL. Mix well.

Secondary standard solution (0.100 µg/mL) of tebuconazole
Pipette 1.00 mL of the 10.0 µg/mL secondary standard solution into a 100-mL volumetric flask. Dilute with acetonitrile to get a concentration of 0.100 µg/mL. Mix well.

Secondary standard solution (0.0100 µg/mL) of tebuconazole
Pipette 5.00 mL of the 0.100 µg/mL secondary standard solution into a 50-mL volumetric flask. Dilute with acetonitrile to get a concentration of 0.0100 µg/mL. Mix well.

Primary stock internal standard (IS) solution of tebuconazole
Prepare the primary IS stock solution of tebuconazole by pipetting 5.00 mL acetonitrile into the pre-weighed (approximately 0.005 g) IS standard vial. Mix well.

Secondary IS solution (10.0 µg/mL) of tebuconazole
Pipette an appropriate volume of the primary IS solution into a 100-mL volumetric flask. Dilute with acetonitrile to get a concentration of 10.0 µg/mL. Mix well.

Secondary IS solution (1.00 µg/mL) of tebuconazole
Pipette 5.00 mL of the 10.0 µg/mL secondary IS solution into a 50-mL volumetric flask. Dilute with acetonitrile to get a concentration of 1.00 µg/mL. Mix well.

Secondary IS solution (0.005 µg/mL) of tebuconazole
Pipette 0.250 mL of the 1.00 µg/mL secondary IS solution into a 50-mL volumetric flask. Dilute with acetonitrile to get a concentration of 0.005 µg/mL. Mix well.

Calibration standard solutions
Prepare working calibration solutions consisting of 0.0250, 0.0500, 0.100, 0.200, 0.500, and 1.00 ppb of tebuconazole by diluting to 50.0 mL with 0.01% formic acid in water. Before bringing the calibration solutions to volume, pipette 1.00 mL of the 0.005 µg/mL IS solution into 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)
0.100	0.005	0.500	1.00	1.00	0.100
0.100	0.005	0.250	1.00	0.500	0.100
0.100	0.005	0.100	1.00	0.200	0.100
0.0100	0.005	0.500	1.00	0.100	0.100
0.0100	0.005	0.250	1.00	0.0500	0.100
0.0100	0.005	0.125	1.00	0.0250	0.100

The primary internal standard stock solution was stored in a freezer. All other solutions were stored in a refrigerator when not in use.

3.5 LC-MS/MS Instrumentation

Samples were analysed using an Agilent 1200 Series HPLC with Sciex® Triple Quad™ 4000 MS/MS. Typical operating conditions for the HPLC and MS/MS are presented in Table 3 and Table 4, respectively.

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. 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.

The analyte, including the IS, was optimized on the Sciex® Triple Quad™ 4000 via infusion. HPLC conditions are presented in Table 3. The MS/MS conditions for the analyte and the IS are listed in Table 4.

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

4.4 Time Required for Analysis

It took one person approximately 1 hour to complete the preparation of one set of 13 samples (one reagent blank, two unfortified matrix control samples, and 10 fortified samples). Time of analysis was approximately 3 hours. Complete analysis of one set, including sample preparation, analysis, and data processing took approximately 1 day.

4.5 Communication with Study Monitor

No communication was made during the course of study between the validation laboratory study director and the study monitor concerning any technical or procedural aspects of the analytical method. E-mails were exchanged regarding method clarification only.

4.6 Method Modification

No major method modifications were made during the course of the study, except that the temperature of the MS ion source was increased to 450°C for improved sensitivity and reduced source contamination.

5.0 CONCLUSIONS

CPS successfully completed the ILV for Bayer Method HW-005-W17-01 (see Appendix 4; "An Analytical Method for the Determination of Residues of Tebuconazole in Drinking and Surface Water Using LC/MS/MS"). Bayer Method HW-005-W17-01 was demonstrated to be suitable for the determination of tebuconazole at the LOQ of 0.050 ppb in water.

6.0 REFERENCES

1. An Analytical Method for the Determination of Residues of Tebuconazole in Drinking and Surface Water Using LC/MS/MS. Diane Beck. February 28, 2017.

2. US Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention. 2012. Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.

3. US Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention. 1996. Residue Chemistry Test Guidelines OPPTS 860.1340: Residue Analytical Method.

4. US Environmental Protection Agency, Office of Compliance Monitoring. 2011. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule, 40 CFR, Part 160.

Table 3 HPLC System Operating Parameters for Bayer Method HW-005-W17-01

HPLC System: Agilent Series 1200 Modular HPLC system
Software: Sciex® Analyst® 1.6.2

LC Conditions
Analytical Column: Phenomenex® Luna® C18(2)-HST, 2.5 µm, 50 × 2.0 mm
Mobile Phase: (A): 0.1% formic acid in water
(B): 0.1% formic acid in acetonitrile

Column Temperature: 40°C
Injection Volume: 50.0 µL
Injection Speed: 200 µL/second
Run Time: 5.0 minutes
Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.00	50	50	500
0.50	50	50	500
2.00	20	80	500
2.10	0.0	100	500
2.50	0.0	100	500
2.60	50	50	500
5.00	Stop		

Retention Time: 1.97 min

Table 4 MS/MS Operating Parameters for Bayer Method HW-005-W17-01

Tandem Mass Spectrometry System, Sciex® Triple Quad™ 4000
Software: Sciex® Analyst® 1.6.2

The following parameters were used for operation of the mass spectrometer for the determination of tebuconazole.

Parameter	Setting
Ion Source:	Turbo Spray (electrospray ionization)
Scan Type:	MRM
Polarity:	Positive
Collision Gas (CAD):	10
Curtain Gas (CUR):	10
Ion Source Gas 1 (GS1):	40
Ion Source Gas 2 (GS2):	40
Ion Spray Voltage (IS):	3500 V
Temperature (TEM):	450°C
Interface Heater (IHE):	ON

Analyte Name	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	DP (V)	CE (V)	EP (V)	CXP (V)
Tebuconazole—Primary	308.2	70.0	100	71	43	10	16
Tebuconazole—Confirmatory	308.2	125.0	100	71	43	10	16
Tebuconazole-$^{13}C_2$-$^{15}N_3$[a]	313.1	75.0	100	71	43	10	16

a Used as an isotopic internal standard for tebuconazole

Appendix 2 Calculations

Residue concentrations were determined using calibration curves that 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:

$Y = MX + B$ with 1/x weighting

where: X is the concentration in ppb
M is the slope
B is the y-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:

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

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

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

where: C_{sp} = spiking solution concentration
V_{sp} = spiking solution volume (0.050 mL)
V = initial sample volume (10 mL)

Recoveries were calculated using the following equation:

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