1.0 SUMMARY

The purpose of this study was to conduct an independent laboratory validation (ILV) of Bayer CropScience analytical method HW-001-S09-01 entitled “An Analytical Method for the Determination of Residues of Tebuconazole In Soil Using LC/MS/MS”, as written [4].

The BCS Analytical Method HW-001-S09-01 was validated using sub-samples of untreated soil from the surrounding site of SynTech Research Laboratory Services, LLC near Stilwell, Kansas. Soil samples were stored in a freezer and warmed to room temperature prior to fortification and preparation for analysis.

In brief, the method used for analysis was as follows:

Soil samples (20 g) were extracted in a microwave extractor with a mixture of methanol/water (7:3,v/v). The extracts were centrifuged to remove fine particulates of the soil. Possible matrix effects of tebuconazole were eliminated by using an internal standard solution of isotopically labeled reference item. Identification and quantitation of the active substance was performed by high performance liquid chromatography using tandem MS/MS detection (LC-MS/MS) in the Multiple Reaction Monitoring (MRM) mode using the following MRM transitions:

Analyte	Quantitation MRM[a]	Confirmation MRM[a]
Tebuconazole	308.15 → 70.16	308.15 → 125.05
Tebuconazole Internal Std (IS)	313.15 → 75.16	NA

[a] All masses reported in amu

The limit of quantitation (LOQ) of the method for the ILV is 10.0 ng/g (ppb) for tebuconazole in soil.

Apparent residues in one control sample was 1.21 ppb for tebuconazole quantitation and 1.15 ppb for tebuconazole confirmation, 44% above and 55% above the calculated method MDLs of 0.838 ppb for tebuconazole quantitation and MDL of 0.741 ppb for tebuconazole confirmation, respectively. The recoveries were not corrected for these residues or interferences. The correlation between the injected amount of substance and the detector response was linear for standards ranging from 0.00 ppb to 200 ppb injection concentration for tebuconazole, with correlation coefficients of >0.99. Sample equivalencies for soil for the calibration standards were 0.0 to 1000 ppb.

Samples were fortified at the LOQ and ten times the LOQ (10xLOQ). In soil, the LOQ level was 10 ng/g (ppb), and the 10xLOQ level was 100 ng/g (ppb).

2.0 INTRODUCTION

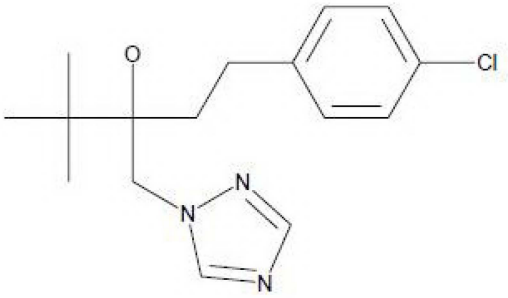
The purpose of this study was to validate the analytical method HW-001-S09-01 for tebuconazole in soil. This study was conducted to satisfy guidelines issued by the US EPA found in OPPTS 860.1340 (c)(6) and PR Notice 96-1 (2,3). Additionally, the independent validation encompassed the guidelines found in EU Council Directive 91/414/EEC with particular regard to section 2 of the European Commission Guidance Document – SANCO/825/00 rev. 7, Directorate General Health and Consumer Protection dated March 17, 2004 (4). This study was conducted in compliance with the EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160.

3.0 MATERIALS AND METHODS

3.1 TEST ITEMS/REFERENCE SUBSTANCES

Only sufficiently characterized and certified substances were used as reference items.

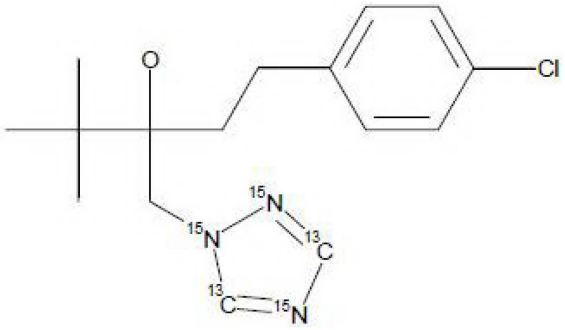
Tebuconazole:



CAS Name:	α-[2-(4-Chlorophenyl)ethyl]-α-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol
CAS Number	107534-96-3
Molecular Formula:	$C_{16}H_{22}ClN_3O$
Molecular Weight:	308 g/mol
ID No.:	K-2213
Purity:	96.8%
Expiration Date:	4/26/23
Date of Analysis:	4/26/13
Storage Conditions:	Refrigerator
Source:	Bayer CropScience, Research Triangle Park

3.2 INTERNAL STANDARD

Tebuconazole-$^{13}C_2$-$^{15}N_3$:



CAS Name:	None
CAS Number	None
Molecular Formula:	$^{13}C_2C_{14}H_{22}Cl^{15}N_3O$
Molecular Weight:	312.78 g/mol
ID No.:	K-2075
Purity:	100%
Expiration Date:	8/13/23
Date of Analysis:	8/13/13
Storage Conditions:	Freezer
Source:	Bayer CropScience, Research Triangle Park

Characterization data for the reference substance (analytical standards) and internal standard are maintained by Bayer CropScience.

The test/reference substance (analytical standard) and internal standard used in this study were procured from Bayer CropScience and stored as directed on “Analytical Standards Chain of Custody” and “Certificate of Analysis” documents. All solutions made from the reference substance (analytical standard) were stored according to the method.

3.3 TEST SYSTEMS

The analytical method HW-001-S09-01 was evaluated in soil from the United States. Sample Chain of Custody (CoC) and analytical results are supplied in the raw data. The soil was collected on March 23, 2017 from a test field at SynTech Research Laboratory Services, LLC in the state of Kansas (KS). The soil sample was classified according to USDA specifications and the characteristics are summarized below (values are not verified):

Description	Kansas Soil (KS)
% Moisture @ 1/3 Bar (Initial)	27.5
% Moisture @ 1 Bar (Analysis)	5.27
pH (1:1 soil:water ratio)	5.5
Organic carbon[a]	0.99
Organic matter (%)	1.7
Cation Exchange Capacity (CEC) (meq/100 g dry soil)	8.1
Maximum water holding capacity (g water/100 g dry soil)	NR
USDA Textural Description (Fraction %)	
Clay (<0.002 mm)	14
Silt (0.002 – 0.050 mm)	57
Sand (0.050 - 2.000 mm)	29
Soil type	Silt Loam

[a] Organic carbon = Organic matter ÷ 1.72 NR = Not reported

3.4 SAMPLE PREPARATION AND EXTRACTION

Bayer Analytical Method HW-001-S09-01 was used for the analysis of tebuconazole. The preparation of the sample residues were as follows:

Soil samples (20 g) were weighed into Teflon pressure vessels, fortified, allowed to sit for a minimum of 5 minutes, washed with a mixture of methanol/water (7:3, v/v), capped, and extracted near 90 °C for 25 minutes in a microwave extractor. After cooling, the solutions were fortified with internal standard for tebuconazole, and mixed well. Approximately 1.5 mL of the extract was transferred to a 2 mL HPLC vial and centrifuged for 10 minutes at 3500 rpm to remove fine particulates of the soil. A 1 mL aliquot of the supernatant was then removed and diluted with 1 mL methanol/water (7:3, v/v), mixed well and then analyzed by LC/MS/MS. Possible matrix effects of tebuconazole were eliminated by using an internal standard solution of isotopically labeled reference item.

Two product ions were selected for monitoring; one ion mass transition served as quantitation and the other ion mass transition was used for confirmation. The mass transitions for both the quantitation as well as the confirmation are presented below:

Analyte	Quantitation MRM[a]	Confirmation MRM[a]
Tebuconazole	308.15 → 70.16	308.15 → 125.05
Tebuconazole Internal Std (IS)	313.15 → 75.16	NA

[a] All masses reported in amu

3.5 INSTRUMENTATION

Standard Method

The HPLC conditions employed were as follows:

HPLC Systems:
Thermo UltiMate 3000 XRS Autosampler, Pump, and Column Oven

HPLC Analytical:
Column: Phenomenex Gemini C18 sorbent, 110Å pore, 50 mm length x 2 mm i.d., 5 µm particle, Ser. No. 598571-2

Mobile phase:
Solvent A: 5 mm Ammonium Acetate in Water
Solvent B: Methanol

Gradient:

Time (min)	% Solvent A	% Solvent B
0.0	80	20
0.5	80	20
2.0	5	95
4.0	5	95
4.01	80	20
6.0	80	20

Divert Valve: Programmed to divert LC flow from column to waste (bypassing detector) from 0 to 1 minute. LC flow is directed to detector during the 1 to 5.5 minute window, then to waste from 5.5 to six minutes.

Flow rate: 0.200 mL/min

Column Temperature: Ambient (~19 ºC)

Injection Volume: 10 µL

Retention Times: Tebuconazole ~4.07 min.

The MS/MS conditions employed were as follows:

MS System: TSQ Quantiva with LCquan 2.9
Interface: Electrospray (H-ESI)
Ionization Mode: Positive (+)
Acquisition mode: MRM (Multiple Reaction Monitoring)

Ion Spray Voltage (IS): 3500 V
Ion Transfer Tube Temp: 325 ºC
Vaporizer Temp: 325 ºC
Sheath Gas (Arb): 35

Aux Gas (Arb):	10
Sweep Gas (Arb):	0
CID Gas (mTorr):	1
Resolution Q1:	0.7
Resolution Q3:	0.7

Analyte (Q = Quant; C = Confirm)	Q1 Mass	Q3 Mass	Dwell Time (ms)	CE (V)
Tebuconazole Q	308.15	70.16	75	25
Tebuconazole C	308.15	125.05	75	37
Tebuconazole-IS Q	313.15	75.16	75	25

Example LC/MS/MS conditions are presented in Appendix 3.

3.6 CALCULATIONS

The example calculations displayed below were used by the laboratory performing the independent laboratory validation. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Tebuconazole residues were quantified using internal standard linear regression analysis. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by 1/x weighted linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL using Thermo LCQuan Software (Version 2.9), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients for slope, M, and intercept, B, for each analytical set. The calibration standards were fit to the linear equation:

$$Y = MX + B$$

where: X is the concentration of the reference standard in ng/mL[a]
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

[a] As the reference standards and samples contain the same internal standard concentrations this value may be omitted from the calculation

The equation shown below is used to calculate of tebuconazole residues.
After regression coefficients were calculated, residues in ng/g(ppb) are determined using the following equation,

$$\text{Tebuconazole found (ng/g)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

Where: V_1 = 50 mL V_3 = 2 mL
W = 20g V_2 = 1 mL

This soil method has a Dilution Factor (D)—or "sample equivalency conversion"—of approximately five (5) relative to the injection concentration. The fortification values were entered for a regression result (X) to be the native injection concentration, and Excel software was used to calculate the amount of tebuconazole in ppb (ng/g) for each sample by multiplying the LCQuan result by the individual Dilution Factors, then the percent recovery for the spiked samples.

The percent recovery was calculated as follows:

$$\text{Recovery } \% = \frac{R}{T} X 100$$

Where: R = ppb of target analyte found in fortified samples
T = theoretical ppb in fortified sample

4.7 TIME REQUIREMENTS

A single analyst completed a sample set consisting of 13 samples in two to three hours with HPLC-MS/MS analysis performed within six hours.

4.8 PROTOCOL/SOP/METHOD DEVIATIONS

- The method was modified with a confirmation transition; the modification is Appendix 7.
- The original column listed in the method is discontinued at the vendor, so a substitute, equivalent column was approved by the Client.
- The original Internal Standard labeling was 15N3 substituted (M+3), and is no longer used; the Client supplied 15N3, 13C2 (M+5) Internal Standard, and the acquisition method was adjusted to the extra mass.
- The protocol calculation equation does not substitute control residues, per the EPA guidelines; the original method called for subtraction.
- The first set failed with low recoveries. The analyst noted control temperatures were below 75 °C during the microwave extraction. Analyst investigation determined the temperature probe and cap had a bad seal. The second set corrected this, and control temperatures registered 85+ °C during the microwave heating, and recoveries passed.
- There was one Control Sample in the validation set that had residues detected above MDL, but still below 30% of the LOQ.

5.0 CONCLUSION

SynTech Research Laboratory Services, LLC successfully independently validated Bayer CropScience analytical method HW-001-S09-01 “An Analytical Method for the Determination of Residues of Tebuconazole In Soil Using LC/MS/MS”[4], with approved modifications. The method was validated on the second validation set.

The method was demonstrated to be suitable for the determination of tebuconazole in soil— from sites in the United States in the state of Kansas (KS). LOQ in soil 10 ng/g (ppb) was detected. Tebuconazole was not found in untreated control (UTC) samples in excess of the Limit of Detection (LOD), defined as one third of the LOQ (3.33 ppb).

These data demonstrate the suitability and the repeatability of the method, confirming analytical method HW-001-S09-01 as an acceptable means to quantify tebuconazole in soil.

6.0 ARCHIVING

A copy of the analytical report, analytical raw data, computer generated listings of raw data and supporting documentation will be retained in the Archives of SynTech Research Laboratory Services, LLC, 17745 South Metcalf Ave. Stilwell, KS 66085 (or at a location specified by the test facility). All originals of the afore-mentioned documents and facility records associated with this study will be sent to the Sponsor to be retained under

Notebook Number RAHW0037 at Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, NC 27709.

7.0 REFERENCES

1. U.S. EPA Ecological Effects Test Guidelines OCSPP 850.6100 Data Reporting for Environmental Chemistry Methods, Public Draft, January 2012.

2. a) U.S. EPA Residue Chemistry Test Guidelines OCSPP 860.1340 Residue Analytical Method, August 1996.
b) EU Council Directive, 91/414/EEC, Section 2 of European Commission Guidance Document –SANCO/825/00 rev. 7, Directorate General Health and Consumer Protection, March 17, 2004.

3. U.S. Environmental Protection Agency, Office of Compliance Monitoring. 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule, 40 CFR, Part 160. Federal Registry, Vol. 54, No. 158: pp. 34052-34074.

4. Netzband, D., Analytical Method HW-001-S09-01: “An Analytical Method for the Determination of Residues of Tebuconazole In Soil Using LC/MS/MS”, 2009.

Appendix 3. Typical HPLC-MS/MS Parameters

Analytical Condition log TQH-Q1-0276 (Quantiva)

Study ID: 007SRUS17R0077
Sequence ID: JDD170523A

Instrument ID: TSQ Quantiva Serial # TQH-Q1-0276
Autosampler ID: Dionex Ultimate 3000 XRS Autosampler Serial # 330573
Pump ID: UltiMate 3000 XRS Serial # LPG-3400-XRS
Column Heater: UltiMate 3000 Serial # 6002689
Quantitation Software Version: LCquan 2.9

Column make: Phenomenex Gemini C18
Column Measurements: 50 x 2.0 mm, 5 µm
Column Temperature (°C): Ambient (~19 °C)

Injection Volume (µL): 40
Loop Volume (µL): 10
Aqueous Mobile Phase ID: 5 mM Ammonium Acetate in Optima water
Oganic Mobile Phase ID: MeOH
Syringe Wash Solvent 2: 1:9 ACN:Water
Syringe Wash Solvent 1: 1:1 MOH:ACE

HPLC Program

Time (min)	% Aqueous	% Organic	Flow Rate (mL/min)
0.0	80	20	0.2
0.5	80	20	0.2
2.0	5	95	0.2
4.0	5	95	0.2
4.01	80	20	0.2
6.0	80	20	0.2

Flow Rate Split Ratio: 1:4 (10 µL to instrument)

Mass Spectrometer Conditions

Compound ID	Tebuconazole Quantitation	Tebuconazole Confirmation	Tebuconazole Internal Standard
Ionization Mode		H-ESI	
Polarity		Positive	
Q1 Mass (m/z)	308.15	308.15	313.15
Q3 Mass (m/z)	70.16	125.05	75.16
Q1 Mass (FWHM)		0.70	
Q3 Mass (FWHM)		1.0	
Collision Energy (v)	25	37	25
Collision Gas Pressure (arbitrary unit)		2.00	
Sheath Gas (arbitrary unit)		35	
Auxiliary Gas (arbitrary unit)		10	
Spray Voltage (v)		3500	
Capillary Heater (°C)		235	
Vaporizer Needle (°C)		275	
Sweep Gas (arbitrary unit)		0.00	
Chrom Filter (sec)		5	
Scan Width (m/z)		0.70	
Scan Time (sec)		0.70	
Tube Lens (v)		68	

Appendix 4 Example Calculations

All residues were quantified using linear regression analysis with LCQuan Software, a computer-programmed data capturing system. The LCQuan Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. For tebuconazole, a calibration curve was generated by linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL.

Tebuconazole

The tebuconazole standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area/isotopic peak area ratio

The slope and intercept were obtained from the calibration curve generated by LCQuan, and are presented in Appendix 1. The calibration points were weighted 1/x to provide better fit near the limit of detection.

The example is for the calculation of tebuconazole quantitation residues for sample [Tebu-Soil-ILV-LOQ-06] (set JDD170523A) which was fortified to 10 ppb of tebuconazole. This chromatogram is presented in Appendix 2.

After regression coefficients were calculated, the residue in ppb was determined. The ppb of tebuconazole in soil was calculated using the following equation:

$$\text{Tebuconazole found (ppb)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume } (V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

Tebuconazole Native Peak Area		Tebuconazole IS Peak Area	
928,723		2,537,792	
[Y]	[M]	[B]	[D]
0.36596	0.188046	0.0011695	5.0005
W	V1	V2	V3
19.998 g	50 mL	1.0 mL	2.0 mL

Using the data from the table and the above equations:

$$\text{Dilution Factor (D)} = \frac{50\ \text{mL} * 2\ \text{mL}}{19.998\ \text{g} * 1\ \text{mL}} = 5.0005$$

$$\text{Tebuconazole found} = \frac{(0.36596 - [0.0011695])}{0.188046} \times 5.0005 = 9.70\ \text{ppb}$$

Therefore sample Tebu-Soil-ILV-LOQ-06 contains 9.70 ppb tebuconazole which agrees with the value reported in the raw data.

The percent recovery was calculated in Excel as follows:

$$\text{Recovery \%} = \frac{R}{T} X 100$$

Where: R = ppb of target analyte found in fortified samples
T = theoretical ppb in fortified sample

9.70 ppb of tebuconazole was detected in soil sample [Tebu-Soil-ILV-LOQ-06] (R); and the sample was fortified to 10 ppb tebuconazole (T).

Therefore:

$$\text{Tebuconazole \% Recovery} = \frac{9.70}{10.0} \times 100 = 97\%$$