Appendix 4   Bayer Method HW-005-W17-01

Title

An Analytical Method for the Determination of Residues of Tebuconazole in Drinking and Surface Water Using LC/MS/MS

- Analytical Method -

Test Substance

Tebuconazole

Guideline Number

US EPA Test Guideline OCSPP 860.1340: Residue Analytical Method

OECD Guidance Document on Pesticide Residue Analytical Methods, Series on Testing and Assessment Document 72 and Series on Pesticides: Document 39, August 2007 (OECD Guideline, ENV/JM/MONO (2007) 17, Aug 13, 2007)

PMRA Residue Chemistry Guidelines, Regulatory Directive 98-02, Section 3, Residue Analytical Method, June 1998

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Completion Date

2017-02-28

Test Facility	**Sponsor**
Bayer CropScience	Bayer CropScience
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Bayer Method Number	**Activity ID**
HW-005-W17-01	RAHW0038

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

1.0 SUMMARY

A previous Bayer analytical method - 01387/M001[1] was developed to determine the residues of various compounds including tebuconazole in drinking and surface water using LC/MS/MS. This method was modified to use an isotopic internal standard and the use of matrix-matched standards omitted. The revised method was assigned a method number of HW-005-W17-01.

Sample aliquots are amended with formic acid and an isotopic internal standard, injected directly into the LC/MS/MS and analyzed with quantification based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of tebuconazole in water at a target limit of quantitation (LOQ) of 0.05 ng/mL, but this LOQ can be adjusted as required.

2.0 BACKGROUND

Tebuconazole is a fungicide currently produced by Bayer CropScience. The analytical method presented in this report is designed to measure tebuconazole in water matrices using isotopically-labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

Functional equivalents may be substituted.

- Various general laboratory glassware and utensils.
- Calibrated variable pipettors and tips
- Glass vials, 10+ mL capacity
- Luna 2.5 µm C18(2) 100 Å, 50 x 2 mm (Part No: 00B-4446-B0) or equivalent
- 2 mL autosampler vials
- ABSciex 5500 chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Shimadzu HPLC pumps, Shimadzu oven and a CTC PAL autosampler, and Analyst 1.6.2 data collection software (ABSciex)

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted.

- Methanol, LC/MS Grade (Fisher Scientific A456-1)
- Acetonitrile (Fisher Scientific, Optima grade. A996-4)
- Water (Fisher Scientific, Optima grade. W7-1)
- Formic acid, 99.5+% LC/MS grade (Fisher Scientific, Optima grade. A117-50)
- Ammonium formate, LC/MS grade (Fisher Scientific, Optima grade. A11550)
- 0.01% formic acid: prepare by adding 0.1 mL formic acid to 1000 mL of laboratory grade water
- 1% formic acid: prepare by adding 1 mL formic acid to 100 mL of laboratory grade water

5.0 PREPARATION OF STANDARD SOLUTIONS

The native reference standard used in this method is tebuconazole. The isotopic internal standard (IS) required for this method is tebuconazole-$^{13}C_2,^{15}N_3$. These standards may be obtained from Bayer CropScience, 2 T. W. Alexander Drive, Research Triangle Park, North Carolina, 27709. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

NOTE: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Volumetric glassware and calibrated pipets should be used in the preparation of all analytical standards. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standards

Reference standards and internal standard primary solutions are prepared as shown below.

Table 1. Primary reference (native) standard solution preparation

Reference Standard	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Tebuconazole	~10	100	ACN	~100

Table 2. Primary internal standard solution preparation.

Reference Standard	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Tebuconazole-$^{13}C_2,^{15}N_3$	~5	50	ACN	~100

5.2 Secondary Standard Solutions

Secondary reference and internal standard solutions are prepared from the primary standard solutions as shown below. Take the appropriate aliquot of each of the primary standard solution to give the required secondary standard concentration.

Table 3. Secondary reference standard solution preparation

Compound	Standard Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Secondary Standard Concentration (µg/mL)	Solvent
Tebuconazole	~100	~5	50.0	10.0	ACN
Tebuconazole	10.0	5.0	50.0	1.0	ACN
Tebuconazole	1.0	5.0	50.0	0.1	ACN
Tebuconazole	0.1	5.0	50.0	0.01	ACN
Tebuconazole	1.0	0.25	50.0	0.005	ACN

Table 4. Secondary internal standard solution preparation

Compound	Standard Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Secondary Standard Concentration (µg/mL)	Solvent
Tebuconazole-$^{13}C_2,^{15}N_3$	~100	~5	50.0	10.0	ACN
Tebuconazole-$^{13}C_2,^{15}N_3$	10.0	5.0	50.0	1.0	ACN
Tebuconazole-$^{13}C_2,^{15}N_3$	1.0	0.25	50.0	0.005	ACN

Secondary standards should be refrigerated when not in use.

5.3   Calibration Standards

Note: Additional standards may be prepared when necessary; however, the concentration of internal standard must remain the same in all calibration standards. Calibration solutions are diluted to volume in 0.01% formic acid in water.

Table 5. Calibration standard solution preparation

Concentration of Native Standard Solution used for dilution (µg/mL)	Concentration of Internal Standard Solution used for dilution (µg/mL)	Aliquot Native Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Native in Calibration Solution (ng/mL)	Concentration of IS in Calibration Solution (ng/mL)
0.10	0.005	0.5	1.0	50.0	1.0	0.10
0.10	0.005	0.25	1.0	50.0	0.50	0.10
0.10	0.005	0.1	1.0	50.0	0.20	0.10
0.010	0.005	0.5	1.0	50.0	0.10	0.10
0.010	0.005	0.25	1.0	50.0	0.05	0.10
0.005	0.005	0.25	1.0	50.0	0.025	0.10

Calibration standards should be stored in a refrigerator when not in use.

6.0   SAMPLE PREPARATION

Fortified recovery samples are prepared by spiking a known level of the appropriate standard(s) into a sample. A control sample should also be prepared, using the same samples as the fortified sample, to determine the residue levels, real or apparent, found in this sample and the % recovery calculated as described in Section 8.1.

1. Transfer a 10.0 mL aliquot of a water sample to a suitably sized stoppered container.

    Remark: Add the corresponding amount of each analyte for the fortified recovery sample (e.g. 0.050 mL of a 0.01 µg/mL analyte mixture at LOQ level of 0.05ng/mL).

2. Add 200 µL of the 0.005 µg/mL internal standard solution (Table 4) to the water sample and mix well.

3. Add 0.1 mL of 1% formic acid in water to the water sample, cap vial and mix.

4. Transfer an aliquot of the water sample to a 2 mL autosampler vial. The sample is ready for analysis by LC/MS/MS.

7.0   ANALYSIS BY LC/MS/MS

7.1   Analytical Procedure

1. Using the recommended procedures listed below; analyze a minimum of five calibration standard solutions (if necessary, additional standard solutions may be added).

2. Analyze an aliquot of each of the analytical samples.

    Note: Up to 20 sample analyses can be made after the analysis of the standard solutions.

3. Repeat Step 1.

4. When necessary analyze additional samples and standard solutions. Always finish the procedure with a set of calibration solutions

7.2   HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. As the HPLC column ages, the retention times of the analytes may change.

Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 0.1% formic acid in ACN
Oven: 40 ºC
HPLC column: Luna 2.5 µm C18(2) 100 Å, 50 x 2 mm ((or equivalent)
Injection volume: 50 µL (Adjust as needed)

Time (min)	Mobile Phase B%	Flow rate µL/min
0.5	50	500
2.0	80	500
2.1	100	500
2.5	100	500
2.6	50	500

Analyte	Approx Retention Time (min)
Tebuconazole	1.45

7.3   Mass Spectrometer Conditions

The MS/MS instrument is operated in the multiple reaction monitoring mode (MRM). Precursor ions are selected and product ions created by collision-induced dissociation.

Two product ions per analyte are listed in Table 6 one product ion (MRM-transition) serving for quantitation and the second ion to be used, only if required, to confirm the presence of any detected residues.

Note: The following recommended conditions were used on an ABSciex 5500 instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

Ionization Mode:	Electrospray ionization (ESI) interface
Curtain Gas (CUR)	10
Collision Gas (CAD)	9
Ion Spray Voltage (IS)	3500
Temperature (TEM)	150
Ion Source Gas 1 (GS1)	50
Ion Source Gas 2 (GS2)	50

Table 6. Mass Spectrometer Parameters

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	CE	CXP	DP	EP
Tebuconazole (Quantitation MRM)	Pos	308	70	100	27	14	100	10
Tebuconazole (Confirmatory MRM)	Pos	308	125	100	27	14	100	10
Tebuconazole IS	Pos	313	75	100	27	14	100	10

8.0   Calculation of Results

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Residue concentrations were determined using calibration curves which were generated after each analysis using ABSciex Analyst 1.6.2 software using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

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

After regression coefficients were calculated, the residue in ng/mL was determined using the following equation,

$$\text{Residue (ng/mL)} \quad = \quad \frac{(Y-B)}{M}$$

Residue levels beyond the calibration curve: the calibration curve can be extended to cover the unknown sample. If neither option is viable, contact the development laboratory for instructions on how to proceed.

8.1   Fortification Experiments

Recovery (fortification) experiments may be performed as needed to monitor method efficiency and reproducibility. Recovery experiments are intended to be used for data collection methods or establishing and validating method efficiency and are prepared by adding a known amount of native standard solution to a sample aliquot and preparing the sample for analysis as described in Section 6.

With each sample set, prepare and analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \text{ x } 100$$

Where: R = ng/mL of target analyte found in fortified (recovery) sample
S = ng/mL of target analyte found in control sample, real or apparent
T = theoretical ng/mL in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Recovery samples are fortified prior to extraction at the LOQ of 10 ng/g or other appropriate level with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

9.0   REFERENCES

No.	Doc. No.	Report No.	Author(s). Title. Year.
1	Method-No. 01387/M001	MR-14/053	**Krebber, R., Ruttmann, F., Leppelt, L.** Modification M001 of the Analytical Method 01387 for the Determination of Various Pesticides in Drinking and Surface Water by HPLC-MS/MS

Appendix 1 Test and Reference Substances

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

Code Name:	Tebuconazole
Synonyms:	HWG 1608
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:	307.82 g/mol
Chemical Structure:	

65
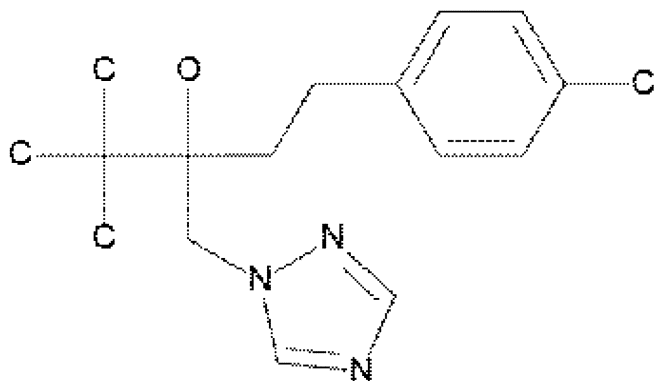
Bayer CropScience

Code Name: Tebuconazole-$^{13}C_2$, $^{15}N_3$
CAS Number: Not available
Molecular Formula: $^{13}C_2C_{14}H_{22}Cl^{15}N_3O$
Molecular Weight: 312.78 g/mol
Chemical Structure: