1.0 Introduction

The Critical Path Services, LLC (CPS), Method 07102014-01 was developed to determine terbacil and its three metabolites in water using LC-MS/MS. The limit of quantitation for the method is 0.01 µg/mL in water. This method satisfies OECD Guidance Document ENV/JM/MONO (2007)17, US EPA OPPTS 860.1340, and EU guidelines SANCO/3029/99 rev. 4 and SANCO/825/00 rev. 8.1.

This method was subsequently validated at CPS by an independent team. The validating team did not discuss the method with the development team in any fashion. Although the same resources of a general, non-specific nature (shakers, balances, glassware, etc.) may have been used by the validating team, such equipment is not considered to be unique to the performance of this method. A significant and variable resource (HPLC-MS/MS) was not shared. A different chromatographic system, the most significant source of possible variance for this type of method, was used in the validation study. The study directors for both the development and validation studies did report to the same supervisor, but the execution and performance of this method were not discussed. Contact was made with the sponsor by the study director of the validation study as duly noted in the independent laboratory validation report.

2.0 Materials and Equipment

2.1 Safety

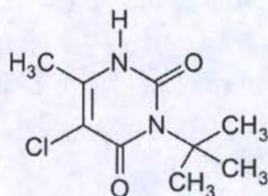
The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good laboratory practice. Details are given in the Safety Data Sheets of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood.

Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

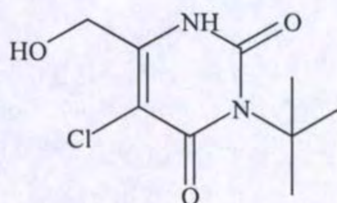
2.2 Test Articles

Compound name:	Terbacil
Chemical name:	3-*tert*-butyl-5-chloro-6-methyluracil
CAS number:	5902-51-2
Molecular formula:	$C_9H_{13}ClN_2O_2$
Molecular weight:	216.7
Exact mass:	216.1
Storage condition:	Ambient

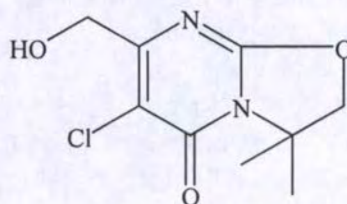
Structure:



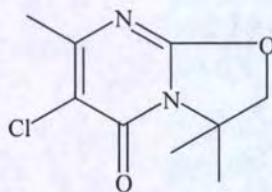
Compound name:	Terbacil Metabolite A
Chemical name:	3-*tert*-butyl-5-chloro-6-hydroxymethyl-uracil
CAS number:	25546-02-5
Molecular formula:	$C_9H_{13}ClN_2O_3$
Molecular weight:	232.7
Exact mass:	232.1
Storage condition:	Ambient
Structure:	



Compound name:	Terbacil Metabolite B
Chemical name:	6-chloro-7-(hydroxymethyl)-3,3-dimethyl-4-hydro-2H-1,3-oxazolidino[3,2-a]pyrimidin-5-one
CAS number:	34138-55-1
Molecular formula:	$C_9H_{11}ClN_2O_3$
Molecular weight:	230.6
Exact mass:	230.0
Storage condition:	Ambient
Structure:	



Compound name: Terbacil Metabolite C
Chemical name: 6-chloro-3,3,7-trimethyl-4-hydro-2H-1,3-oxazolidino[3,2-a]pyrimidin-5-one
CAS number: 34112-90-8
Molecular formula: $C_9H_{11}ClN_2O_2$
Molecular weight: 214.6
Exact mass: 214.1
Storage condition: Ambient
Structure:



2.3 Reagents

HPLC-grade water:	HPLC-grade, EMD, or equivalent
Acetonitrile (ACN):	HPLC-grade, EMD, or equivalent
Formic acid:	Reagent grade (≥99%), Sigma Aldrich®, or equivalent

2.4 Equipment

HPLC column:	Phenomenex Luna C18 (2), 4.60 × 75 mm, 3.0 µm particle size
HPLC system:	Agilent Series 1200, or equivalent
Autosampler:	Agilent Series 1200, or equivalent
Column heater:	Agilent Series 1200, or equivalent
Mass spectrometer:	Sciex 4000 triple quadrupole, or equivalent

Mettler Toledo analytical balance, or equivalent
VWR® vortex mixer, or equivalent
Adjustable manual and electronic pipettes of multiple volumes
Miscellaneous glassware (Kimax®, VWR®, and Fisher Scientific) for preparation of standards, solutions, and mobile phases, etc.
Branson Ultrasonic Cleaner, or equivalent
Supelco SPE manifold, or equivalent
15-mL polypropylene centrifuge tubes
Beckman Coulter Allegra® X-22R centrifuge, or equivalent
Agilent Bond Elut C18 cartridge, 500 mg, 3 cc, part no. 52102028
Agilent glass HPLC vials, or equivalent

3.0 Solutions

3.1 Preparation of Final Sample Solution

Measure 400 mL of ACN and 600 mL of water with a graduated cylinder, and pour both solvents into a 1-L glass reagent bottle. Cap the glass bottle and mix the contents well. Store the solution at room temperature. Expiration is three months.

3.2 Preparation of Mobile Phases for HPLC

Mobile Phase (A): water:formic acid 1000:1(v/v)
Measure 1000 mL of HPLC-grade water with a graduated cylinder, and pour into a 1-L mobile phase bottle. Add 1.00 mL of formic acid to the bottle, and mix well. Mobile phase should be prepared monthly.

Mobile Phase (B): ACN:formic acid 1000:1(v/v)
Measure 1000 mL of HPLC-grade ACN with a graduated cylinder, and pour into a 1-L mobile phase bottle. Add 1.00 mL of formic acid to the bottle, and mix well. Mobile phase should be prepared monthly.

3.3 Preparation of Reference Standard Stock Solutions

Prepare stock solutions using glass volumetric flasks and pipettes. Correct the solution concentration appropriately by purity. Store all solutions in a refrigerator set to maintain 2 to 8°C. An expiration date of 12 months is recommended for the stock standard solution.

Primary Stock Solutions
Weigh approximately 10.00 mg of reference standard using an analytical balance, record the actual weight, and calculate the adjusted weight by factoring in the purity. Quantitatively transfer the weighted reference standard to a 50.0-mL volumetric flask, and adjust the flask contents to volume with ACN. Mix the contents well with 5 minutes of sonication with periodic vortexing. Calculate the exact concentration of the stock solution. The target concentration for the primary stock solution is 200 µg/mL. Prepare primary stock solution separately for each analyte.

3.4 Preparation of Fortification Standard Solutions

Fortification standards should be prepared in 40:60 ACN:water (v/v) solution and stored in a refrigerator set to maintain 2 to 8°C. An expiration date of three months is recommended for the fortification standard solutions.

Fortification standard 10.0 µg/mL is prepared by pipetting 2.50 mL of each primary stock solution into a 50-mL volumetric flask. The volume is brought to the mark with 40:60 ACN:water (v/v) solution.

Fortification standard 1.00 µg/mL is prepared by pipetting 5.00 mL of fortification standard 10.0 µg/mL into a 50-mL volumetric flask. The volume is brought to the mark with 40:60 ACN:water (v/v) solution.

Calculate the transfer volume based on the primary stock solution's actual concentration, so that the fortification standard will have the exact concentration of 10.0 µg/mL for all analytes. For example, if a primary stock solution has an analyte concentration of 197 µg/mL, then 2.54 mL should be transferred to the 50.0-mL volumetric flask.

3.5 Preparation of Calibration Standard Solutions

Calibration standards should be prepared in 40:60 ACN:water (v/v) solution and stored in a refrigerator set to maintain 2 to 8°C. An expiration date of three months is recommended for the calibration standard solutions.

Calibration Standard Solutions:

Calibration Standard ID	Source Solution Conc. (µg/mL)	Source Solution Volume (µL)	Final Volume (mL)	Calibration Standard Conc. (ng/mL)
Std 1	1.00	0.0500	50.0	1.00
Std 2	1.00	0.100	50.0	2.00
Std 3	1.00	0.250	50.0	5.00
Std 4	1.00	0.500	50.0	10.0
Std 5	1.00	1.00	50.0	20.0
Std 6	1.00	2.50	50.0	50.0

4.0 Analytical Procedure

4.1 Sample Preparation Procedure

1. Pipette 3.00-mL aliquots of water sample into a 15-mL centrifuge tube.
2. To prepare fortification samples:
   For limit of quantitation (LOQ; 0.0100 µg/mL): Spike the control water sample with 30.0 µL of fortification standard containing terbacil and its three metabolites at 1.00 µg/mL.
   For 10× LOQ (0.100 µg/mL): Spike the control water sample with 30.0 µL of fortification standard containing terbacil and its three metabolites at 10.0 µg/mL.
3. Cap the sample tube and vortex for a few seconds. For dirty samples, centrifuge for 5 minutes at 4000 rpm, if necessary.
4. Place an Agilent C18 cartridge (500 mg, 3cc, part no. 52102028) on an SPE manifold. Condition the cartridge with 5.00 mL of ACN followed by 3.00 mL of water.
5. Load 3.00 mL of sample. Rinse the sample tube with 3.00 mL of water, then add rinsate to the cartridge.

6. Elute the cartridge with 5.00 mL of ACN and collect the eluent into a 15-mL centrifuge tube.

7. Bring the total sample volume to 6.00 mL with ACN and mix.

8. Pipette 0.900 mL of water and 0.600 mL of sample into an HPLC vial (2.5× dilution), and mix the contents for LC-MS/MS analysis.

Note:
The samples should be refrigerated if instrument analysis is not performed on the same day. The final sample extract stability should be evaluated during the course of the study.

4.2 LC-MS/MS Method

The following MS settings as provided should be used as general guidelines only. For optimal results, tuning for each analyte should be performed by the analyst.
For the best sensitivities, terbacil and metabolite A are analyzed in negative mode, and metabolites B and C are analyzed in positive mode. Therefore, samples may need to be analyzed twice if Sciex 4000 LC-MS/MS is used.

HPLC Conditions:

Column:	Phenomenex Luna C18 (2), 4.60 × 75 mm, 3.0 μm particle size
Column Temp:	30°C
Flow Rate:	0.550 mL/min.
Run Time:	5.0 min.
Injection Volume:	10–50 μL
Mobile Phase:	(A) Formic acid:HPLC-grade water 1:1000 (v/v)
	(B) Formic acid:ACN 1:1000 (v/v)

Mobile Phase Gradient Table:

	Positive (Metabolites B & C)		Negative (Terbacil & Metabolite A)	
Time (min.)	**A%**	**B%**	**A%**	**B%**
0.00	60.0	40.0	55.0	45.0
0.20	60.0	40.0	55.0	45.0
1.00	5.00	95.0	5.00	95.0
3.00	5.00	95.0	5.00	95.0
3.10	60.0	40.0	55.0	45.0
5.00	60.0	40.0	55.0	45.0

MS/MS Conditions:

Instrument:	Sciex 4000 triple quadrupole, or equivalent
Ionization Mode:	Electrospray Ionization
Scan Mode:	Multiple Reaction Monitoring (MRM)

Acquisition Ions and Compound Dependent Parameters:

Analyte	Mass Transition	Dwell	DP	CE	CXP	Mode
Terbacil (Primary)	215→159	150	-55.0	-22.0	-1.00	-
Terbacil (Confirmatory)	215→42.1	150	-55.0	-42.0	-5.00	-
Metabolite A (Primary)	231→65.9	150	-85.0	-38.0	-1.00	-
Metabolite A (Confirmatory)	231→201	150	-85.0	-24.0	-17.0	-
Metabolite B (Primary)	231→213	150	76.0	25.0	14.0	+
Metabolite B (Confirmatory)	231→185	150	76.0	31.0	12.0	+
Metabolite C (Primary)	215→161	150	71.0	27.0	10.0	+
Metabolite C (Confirmatory)	217→163	150	71.0	27.0	10.0	+

Typical MS/MS Source Conditions Used:

Ionization Mode:	Turbospray Positive	Turbospray Negative
Scan Type	MRM	MRM
Resolution Q1	unit	unit
Resolution Q3	unit	unit
Curtain gas (N_2)	30	30
GS1	60	60
GS2	60	60
CAD gas (N_2)	6	6
Ion Spray (V)	5500	-4500
Temperature (°C)	550	550
EP	10	-10

4.3 System Condition and System Precision Check

The instrument should be conditioned and tested before each analytical run. A solvent blank sample and the lowest calibration standard should be injected several times for contamination check and sensitivity check.

4.4 Calibration Curve

The calibration curve should be prepared as described in Section 3.5.

The calibration curve will be determined from peak area vs. analyte concentration using a linear regression with a weighting factor of 1/x recommended. The calibration standard range in this method is 1.00 to 50.0 ng/mL. For a calibration curve to be acceptable, at least 75% (or a minimum of five calibration points) of the calibration standards must have a back calculated concentration within 100 ± 15% (±20% at the lower limit of quantitation [LLOQ], Std1) accuracy. The curve coefficient of determination (r^2) should be ≥0.990.

4.5 Analytical Batch Setup

The standards should be injected prior to and immediately after a sequence of samples. During the analytical run, the calibration standards should be interspersed with samples. If the sample set is >5, then an occasional standard should be analyzed between samples to demonstrate response stability. A standard that represents a residue of 50–70% of the method's LLOQ for each analyte should be included among those standards injected.

Any sample residue which exceeds the highest calibration standard injected should be appropriately diluted and re-injected with a standard set such that it falls within the linear response range of the standards injected, or injected at a lower volume such that it falls within the linear response.

4.6 Calculation

The standards were fit to the linear equation y = mx + b

Where:
x is the concentration of sample in final extract
m is the calibration line slope
b is the calibration line intercept
y is the native peak area

The linear equation can be rearranged and used to calculate residues as follows:

$$ppm(\mu g/mL) = \frac{X(ng/mL) \times Final\ Volume\ (mL) \times Dilution\ Time}{Sample\ Volume\ (mL) \times 1000 \times Aliquot\ Factor}$$

Where:
Final Volume = 6.00 mL
Sample Volume = 3.00 mL
Dilution Time = any additional dilutions after sample preparation
Aliquot Factor = 0.4

4.7 Untreated Control (UTC) and Recovery Acceptance Criteria

The UTC matrix should be essentially free of any interference at the retention time of the compound(s) of interest. Interferences with peak areas that are <50% at the method detection limit (MDL), or limit of detection (LOD), are considered not significant. Interferences in control matrix with peak areas that are <30% of the anticipated LOQ are considered to have no significant impact on the analysis results. If significant interference is observed during the analysis, appropriate investigation will be performed to find out the root source. If UTC truly contains analyte and the value is >30% of the LOQ, recovery data may be corrected for the value in the control.

The analytical run will be considered acceptable if the mean recovery at each spiking level at or above the LOQ throughout the course of a study is between 70% and 120%, and the relative standard deviation (RSD) of independent measurement of recoveries at each level does not exceed ±20%.

If samples were detected above the highest recovery QC concentrations, a higher level of recovery QC (e.g., 1000× LOQ), which covers the concentration of the highest incurred sample, should be prepared and analyzed in the next analytical run.

4.8 Analysis Time

The methodology is normally performed with a batch of 20 to 30 samples. On average, one chemist can analyze up to 30 samples a period of eight working hours, excluding instrument run time.

4.9 Data Processing and Calculations

Conduct data processing and analysis that is generally consistent with the following procedure. Briefly, conduct chromatographic peak integrations with the LC-MS/MS data system software (e.g., Analyst). Export the raw data with three decimal places (minimum three significant figures) for the concentration.

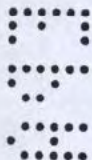
Report concentration results to three significant figures. Report percentages such as recovery, accuracy, and coefficient of variation (CV) to one decimal place. Report coefficient of determination (r) to four decimal places.

Further data analysis should be conducted using Microsoft® Excel®, or other statistical package.

4.10 Modifications and Potential Problems

Testing for ion suppression/enhancement is recommended for the water samples prior to analysis. No significant interferences or ion suppression/enhancement was observed in tap and surface water samples used for the method development.

A different type of C18 cartridge may be used for the analysis. However, a C18 cartridge profile should be performed before use for sample analysis.



6.0 Conclusion

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of residues of terbacil and its three metabolites in water. Only commercially available laboratory equipment and reagents are required. The analysis of 20 to 30 samples can be completed by one chemist in an eight-hour working period. The LOQ of the method is 0.01 μg/mL (ppm) for analysis of terbacil and its three metabolites.

7.0 References

1 Application Note: Comprehensive Quantitation and Identification of Pesticides in Food Samples Using the New Eksigent ekspert™ ultraLC 100 and the New AB Sciex Qtrap® 4500 System. André Schreiber, AB Sciex, Concord, Ontario, Canada. 2012.

2. Application Note: Multiresidue Analysis of 301 Pesticides in Food Samples by LC/Triple Quadrupole Mass Spectrometry. E. Michael Thurman and Imma Ferrer, University of Colorado, Boulder, Colorado; Jerry A. Zweigenbaum, Agilent Technologies, Inc., Wilmington, Delaware. 2008.

3. Study report: Long-Term Field Soil Dissipation of Terbacil Herbicide. Ann Y. Kuo and Luis O. Ruzo, PTRL West, Inc., Richmond, California. 1995.

4. Study Report: Terbacil: Magnitude of the Residue on Watermelon. Janine E. Rose, PTRL West, Inc., Richmond, California. 1999.

5. Study Report: Independent Laboratory Validation for "Determination of Terbacil and Its Three Metabolites in Water Using LC-MS/MS. Bhaskar Malayappan, 2014.