2.0 INTRODUCTION

The objective of this study was to validate CPS Analytical Method 07102014-02, "Determination of Terbacil and Its Three Metabolites in Sediment Using LC-MS/MS." This method passed the ILV for terbacil and its three metabolites in sediment on the second attempt with no major modifications. The target concentrations are based on wet weight of the sediment.

This study was conducted in compliance with EPA Test Guideline OCSPP 850.6100. In addition, this study was conducted in compliance with EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160.

3.0 MATERIALS AND METHODS

3.1 Test Substances

Standard name:	Terbacil
Lot number:	12924-16-16
CPS ID:	14-CPS-Mar20-01
Purity:	98.7%
Date received:	20 March 2014
Expiration date:	17 July 2015
Storage conditions:	Ambient

Standard name:	Terbacil Metabolite A
Lot number:	12924-17-18
CPS ID:	14-CPS-Mar20-02
Purity:	99.8%, 99.5%[1]
Date received:	20 March 2014
Expiration date:	06 September 2014, 13 October 2016[1]
Storage conditions:	Ambient

Standard name:	Terbacil Metabolite B
Lot number:	004
CPS ID:	14-CPS-Jun13-01
Purity:	88.5%
Date received:	13 June 2014
Expiration date:	19 May 2015
Storage conditions:	Frozen

[1] The Certificate of Analysis (COA) for terbacil metabolite A was expired prior to writing the protocol for the ILV. The client was notified, and the client asked that the ILV be performed with the expired COA, indicating that recertification of this metabolite was ongoing. The new COA was received on 19 November 2014. The purity indicated on the old COA is 99.8 ± 0.0% whereas the new COA reports purity as 99.5 ± 0.2%. The change in purity of terbacil metabolite A (new COA) will have no impact on calculations or recovery data.

Standard name:	Terbacil Metabolite C
Lot:	12924-50-23
CPS ID:	14-CPS-Jun13-02
Purity:	99.6%
Date received:	13 June 2014
Expiration date:	19 May 2015
Storage conditions:	Refrigerated

3.2 Test System

The test system used for the validation was sediment.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined on page 3 in CPS Analytical Method 07102014-02 (included in the protocol in Appendix 1) and were documented in the study records (Notebook pages 4–8). Identical or equivalent equipment and materials were used.

3.3.1 Equipment and Apparatuses

Analytical balance (Mettler Toledo), or equivalent
VWR® vortex mixer, or equivalent
Wrist-Action® Shaker Model 75 (Burrell Scientific, LLC), or equivalent
N-EVAP with water bath and thermometer (Organomation Associates, Inc.), or equivalent
Adjustable manual and electronic pipettes of multiple volumes
Miscellaneous glassware (Kimax®, VWR®, and Fisher Scientific) for preparation of standards, solutions, mobile phases, etc.
Ultrasonic cleaner (Branson), or equivalent
50-mL polypropylene centrifuge tubes
1.5-mL self-lock centrifuge tubes
Nylon syringe filter, 0.2 µm (Pall Life Science Corporation), or equivalent
5-mL disposable plastic syringe
Glass wool (VWR®), or equivalent
Powder funnels, 8-cm, or equivalent
Beckman Coulter Allegra® X-22R centrifuge, or equivalent
Glass HPLC vials (Agilent Technologies), or equivalent
Supelclean™ ENVI-Carb™ SPE bulk packing, Supelco, part no. 57210-U. Alternatively, the material can be obtained from a Supelclean™ ENVI-Carb™ cartridge (part no. 57094, 57127-U, etc.).
LC-MS/MS—Agilent 1200 binary pump HPLC system and autosampler, coupled to an Applied Biosystems® API 4000™ mass spectrometer with an electrospray ionization interface

3.3.2 Reagents

Acetonitrile (ACN):	HPLC-grade, EMD, or equivalent
Formic acid:	Reagent grade (≥99%), Sigma Aldrich®, or equivalent
Water:	Milli-Q
Chloroform:	HPLC-grade, EMD, or equivalent
Sodium sulfate, anhydrous (Na_2SO_4):	Reagent grade, EMD, or equivalent.

3.4 Experimental Design

3.4.1 Establishment of the Method

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.

3.4.2 Standard Solution Preparation

Solutions were prepared following the procedures described below.

Preparation of Final Sample Solution
Measure 400 mL of ACN and 600 mL of MilliQ 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.

Preparation of Mobile Phases for HPLC
Mobile Phase (A): Water:formic acid 1000:1 (v/v)
Measure 1000 mL of Milli-Q 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.

Preparation of Reference Standard Stock Solutions
Prepare stock solutions using glass volumetric flasks and pipettes. Store all solutions in a refrigerator set to maintain 2 to 8°C. An expiration date of 12 months is recommended for the reference standard stock solutions.

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 the primary stock solution separately for each analyte.

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.

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

All calibration standard solutions were refrigerated (~4°C) when not in use.

3.4.3 Sample Validation Sets, Fortification, and Extraction Procedure

Sample Validation Sets
An analytical set was prepared for terbacil and its three metabolites. Each analytical set consisted of 13 samples: one reagent blank, two untreated control samples, five untreated control samples fortified at the LOQ (0.0100 µg/g), and five untreated control samples fortified at 10× LOQ (0.100 µg/g).

3.5 Sample Preparation Procedure

Samples were prepared following the procedure described below.

1. Weigh 2.50-g sub-samples into 50-mL centrifuge tubes.
2. Prepare fortification samples:
   For LOQ (0.0100 µg/g): Spike the control sediment sample with 25.0 µL of fortification standard containing terbacil and its three metabolites at 1.00 µg/mL.
   For 10× LOQ (0.100 µg/g): Spike the control sediment sample with 25.0 µL of fortification standard containing terbacil and its three metabolites at 10.0 µg/mL.
3. Add 35 mL chloroform, cap the sample tube, and shake by hand for a few seconds.
4. Place tubes horizontally on the Wrist-Action® Shaker, and shake for ~20 minutes.
5. Centrifuge samples for 5 minutes at 4000 rpm.
6. Transfer supernatant to a clean 50-mL centrifuge tube through a funnel plugged with glass wool and a layer of Na_2SO_4.
7. Start evaporation of the extract using an N-EVAP with a water bath set at ≤40°C.
8. Add 20 mL chloroform to the original sample tube and shake for ~20 minutes on the Wrist-Action® Shaker.
9. Centrifuge for 5 minutes at 4000 rpm and transfer supernatant to a clean 50-mL centrifuge tube through the same funnel.
10. Repeat Steps 8 and 9 once more.
11. Combine extracts with the sample on the N-EVAP and continue evaporation until volume is below 1.00 mL or only water is left.
12. Bring sample volume to 5.00 mL with ACN, vortex, and sonicate.
13. Filter sample through a 0.2-µm nylon syringe filter.
14. Using a spatula, add approximately 25 mg of Supelclean™ ENVI-Carb™ powder to a 1.5-mL self-lock centrifuge tube. Then pipette 1.00 mL of sample into the 1.5-mL centrifuge tube.
15. Vortex sample for a few seconds, then centrifuge at 10,000 rpm for 5 minutes.
16. 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 were diluted (1:1) with 40:60 ACN:water (v/v) solution.

The samples were refrigerated if instrument analysis was not performed on the same day.

Per the original method, terbacil and metabolite A were analyzed in negative mode, and metabolites B and C were analyzed in positive mode. Therefore, samples were analyzed twice on the API™ 4000 LC-MS/MS. Matrix effects were tested prior to the analysis. Since ion suppression was seen for metabolites B and C, those samples were analyzed by an atmospheric pressure chemical ionization (APCI) source, as recommended in the original method.

3.5.1 Sample Processing and Analysis

The samples were analyzed as described in CPS Analytical Method 07102014-02. The samples were analyzed with six calibration standards interspersed with the samples in a sequence. If the sample set was >5, then an occasional standard was analyzed between samples to demonstrate response stability.

3.6 LC-MS/MS Instrumentation

Instrumentation
Agilent 1200 HPLC System (Agilent Technologies)
API 4000™ Tandem Mass Spectrometer, MS/MS (Applied Biosystems®)
HPLC Column: Phenomenex Luna C18 (2), 4.60 × 75 mm, 3.0 µm particle size
Software: Applied Biosystems®, Analyst® version 1.6.2
Refer to Table 3 for the details of the instrument conditions.

3.7 Data Acquisition and Reporting

Peak integration was performed using 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 analytes from the fortified samples were calculated by multi-point calibration.

Recovery results for each analyte were computed for each sample. The equations used for quantification are presented in Appendix 2. A statistical treatment of the data includes the calculation of means, standard deviations (SDs), and RSDs as percentages (%). All statistics were calculated using Microsoft® Office Excel® 2003.

5.0 CONCLUSIONS

CPS successfully completed and independently validated CPS Analytical Method 07102014-02, "Determination of Terbacil and Its Three Metabolites in Sediment Using LC-MS/MS." CPS Analytical Method 07102014-02 is suitable for determining terbacil and its three metabolites in sediments down to a level of 0.0100 µg/g.

6.0 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. Ecological Effects Test Guidelines OCSPP 850.6.100: Environmental Chemistry Methods and Associated Independent Laboratory Validation. USEPA, Office of Chemical Safety and Pollution Prevention (7101), EPA 712-C-001. 2012.

Table 3 LC-MS/MS System Operating Parameters

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:Milli-Q water 1:1000 (v/v) (B) Formic acid:ACN 1:1000 (v/v)

Mobile Phase Gradient Table:

	Positive (Metabolites B and C)		Negative (Terbacil and 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:	API™ 4000 triple quadrupole, or equivalent
Ionization Mode:	Electrospray ionization/APCI
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

APPENDIX 2 CALCULATIONS

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystems® Analyst® software version 1.6.2 using a linear regression with $1/(\text{concentration})^2$ weighting. Further calculations were performed using Microsoft® Office Excel® 2003.

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

Where:
x is the concentration of sample in the 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:

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

Where:
Final Volume = 5.00 mL
Sample Weight = 2.50 g
Dilution Factor = any additional dilutions after sample preparation
Aliquot Factor = 0.4