

SUMMARY

The purpose of this study was to conduct an independent laboratory validation on Wellmark International's study number 4548 entitled "Analytical Method for the Determination of tau-Fluvalinate and Metabolites in Soil, Thatch and Foliage" (Ko et. al., 2015). This ILV study was required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one laboratory, can be independently validated by a second laboratory with no major interaction between the two laboratories. The method was successfully validated on the first attempt in soil, thatch and foliage at the method LOQ (5.00, 10.0 and 20.0 ppb, respectively) and 10X LOQ (50.0, 100 and 200 ppb, respectively) concentration levels, using the method as written.

1.0 INTRODUCTION

Methodology from Wellmark International was validated to quantify the concentration of tau-Fluvalinate and its metabolites (haloanline, 3-phenoxybenzoic acid, anilino acid, diacid and 4-amino-3-chlorobenzoic acid) present in recovery samples prepared in three different matrices (soil, thatch and foliage) on 8 to 16 April 2015. This independent laboratory validation (ILV) study is required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one group, can be independently validated by a second group with no major interaction between the two groups. This method was validated by fortification of soil with tau-Fluvalinate and its metabolites (haloanline, 3-phenoxybenzoic acid, anilino acid and 4-amino-3-chlorobenzoic acid) and thatch and foliage with tau-Fluvalinate and its metabolites (haloanline, 3-phenoxybenzoic acid, anilino acid, diacid and 4-amino-3-chlorobenzoic acid) at concentrations of 5.00, 10.0 and 20.0 ppb (LOQ), respectively, and 50.0, 100 and 200 ppb (10X LOQ), respectively. Recovery samples were extracted once with 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v), placed on an orbital shaker for ten minutes at 250 rpm followed by an aliquot of internal standard added to each sample. Samples were then centrifuged at 3000 rpm for ten minutes. If the supernatant was not sufficiently separated from the matrix, samples were centrifuged for an additional five minutes. If particles were observed after extraction, samples were additionally centrifuged at 13,000 rpm for five minutes. Samples were then analyzed for tau-Fluvalinate and metabolites 3-phenoxybenzoic acid, anilino acid, 4-amino-3-chlorobenzoic acid and diacid (with exception of soil for diacid) using liquid chromatography with mass spectrometry (LC/MS/MS) and for haloanline using gas chromatography with mass spectrometry detection (GC/MS). Recoveries for soil and thatch samples were determined using external standards (not using an internal standard), while the recoveries for foliage samples were determined utilizing an internal standard, triphenyl phosphate (TPP).

The study was initiated on 11 March 2015, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the ILV study was conducted on 8 to 16 April 2015 at Smithers Viscient (SMV), located in

Wareham, Massachusetts. All original raw data and the final report produced during this study are archived at Smithers Viscient at the above location.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled "Tau-Fluvalinate - Independent Laboratory Validation of the Analytical Method for Determination of Tau-Fluvalinate, and its Degradates in Soil, Thatch and Foliage by LC/MS/MS and GCMS", ([Appendix 1](#)). The methods described in this protocol meet the requirements specified in the OCSPP Guidelines 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation ([U.S. EPA, 2012](#)), OSCPP 850.7100: Data Reporting for Environmental Chemistry Methods ([U.S. EPA, 1996a](#)) and OSCPP 860.1340: Residue Analytical Method ([U.S. EPA, 1996b](#)) as well as to satisfy guideline requirements described in OECD ENV/JM/MONO(2007)17 ([OECD, 2007](#)) as well as the guidelines found in EC guidance documents SANCO/3029/99 rev. 4 ([EC, 2000](#)) and SANCO/825/00 rev. 8.1 ([EC, 2010](#)).

2.2 Test Substances and Internal Standard

2.2.1 Test Substances

The test substance, tau-Fluvalinate, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

Name:	tau-Fluvalinate
Synonym:	(RS)-alpha-cyano-3-phenoxybenzyl-R-2-(2-chloro-4-trifluoromethyl)-anilino-3-butanoate
Lot No.:	ARS14-35-MIP2
CAS No.:	102851-06-9
Purity:	91.49% (Certificate of Analysis, Appendix 2)
Expiration Date:	31 July 2015

Upon receipt at Smithers Viscient, the test substance (SMV No. 7555) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, haloanline, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

Name:	haloanline
Synonym:	2-chloro-4-trifluoromethylalanine
Lot No.:	ARS14-57-HALO
CAS No.:	39885-50-2
Purity:	98.70% (Certificate of Analysis, Appendix 2)
Expiration Date:	31 August 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7556) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 3-phenoxybenzoic acid, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

Name:	3-phenoxybenzoic acid
Synonym:	3-PB acid
Lot No.:	ARS14-58-PBA
CAS No.:	3739-38-6
Purity:	99.40% (Certificate of Analysis, Appendix 2)
Expiration Date:	31 August 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7557) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, anilino acid, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

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Name: anilino acid
Synonyms: 2-(2-chloro-4-trifluoromethyl)anilino-3-methylbutanoic acid,
RCAA
Lot No.: ARS14-56-RCAA
CAS No.: 76769-07-8
Purity: 99.57% (Certificate of Analysis, [Appendix 2](#))
Expiration Date: 31 August 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7559) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, FDA Fluvalinate Diacid, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

Name: FDA Fluvalinate Diacid
Synonyms: 2-(2-chloro-4-carboxyl)anilino-3-methylbutanoic acid, diacid
Lot No.: ARS14-60-DIAC
CAS No.: 85236-41-5
Purity: 99.61% (Certificate of Analysis, [Appendix 2](#))
Expiration Date: 31 August 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7560) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 4-amino-3-chlorobenzoic acid, was received on 3 March 2015 from Central Life Sciences, Dallas, Texas. The following information was provided:

Name: 4-amino-3-chlorobenzoic acid
Synonym: ACBA
Lot No.: MKBH9181V
CAS No.: 2486-71-7
Purity: 97.0% (Certificate of Analysis, [Appendix 2](#))
Expiration Date: 30 September 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7561) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identity, maintenance of records on the test substance and archival of a sample of the test substance are the responsibility of the Study Sponsor.

2.2.2 Internal Standard

The internal standard, triphenyl phosphate, was received on 6 March 2015 from Sigma-Aldrich, Allentown, Pennsylvania. The following information was provided:

Name:	triphenyl phosphate
Synonym:	TPP
Batch No.:	BCBK0975V
CAS No.:	115-86-6
Purity:	99.9% (Certificate of Analysis, Appendix 2)
Expiration Date:	Not Listed

Upon receipt at Smithers Viscient, the internal standard (SMV No. 7564) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the internal standard.

2.3 Reagents

1. Acetonitrile: EMD, reagent grade
2. Formic acid: EMD, reagent grade
3. Acetic acid: EMD, reagent grade
4. Purified reagent water: prepared from a Millipore Milli-Q[®] Direct 8 system (meeting ASTM Type II requirements)
5. Dimethyl sulfoxide: BDH, reagent grade
6. Methanol: EMD, reagent grade

2.4 Equipment

1. Instrument: AB Sciex API 5000 mass spectrometer equipped with an AB Sciex Turbo V ESI Ion Spray source, an Acquity Sample Manager autosampler, an Acquity Binary Solvent Manager binary pump, an Acquity Column Compartment column oven and Analyst 1.6 software for data acquisition
2. Balance: Agilent series 6890 gas chromatograph equipped with an Agilent series 7683 autosampler, an Agilent series 7683 injector, an Agilent series 5975 mass selective detector and Agilent ChemStation Version D.00.01 for data acquisition
Mettler Toledo AG245, Mettler Toledo AG285, Mettler Toledo PJ-3000, Mettler Toledo XS205 DR, Sartorius Moisture Analyzer MA-45
3. Shaker table: Orbit Shaker Table 3520, VWR Shaker Table 3500, VWR Shaker Table 3500STD
4. Centrifuge: Beckman Allegra X-12, Eppendorf 5417C
5. Laboratory equipment: volumetric flasks, disposable glass pipets, positive displacement pipets, 50-mL centrifuge tubes, 15-mL centrifuge tubes, 1.5-mL low binding centrifuge tubes, autosampler vials and amber glass bottles with Teflon[®]-lined caps

2.5 Test System

The test systems evaluated in this study were soil, thatch and foliage which were collected from EPA Region 10 located in Sutter County, California. The soil was received on 9 March 2015 from Central Life Sciences, Dallas, Texas and was determined to have a moisture content of 11.27% prior to testing using a Sartorius MA-45 moisture analyzer. The thatch and foliage were also received on 9 March 2015 from Central Life Sciences, Dallas, Texas.

2.6 Preparation of Stock Solutions

A 1000 mg/L primary stock solution was typically prepared by placing 0.0274 g of tau-Fluvalinate (0.0251 g as active ingredient) and bringing it to a volume of 25.0 mL with

0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1060 mg/L primary stock solution was typically prepared by placing 0.0107 g of haloanline (0.0106 g as active ingredient) and bringing it to a volume of 10.0 mL with 0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1000 mg/L primary stock solution was typically prepared by placing 0.01008 g of 3-PB acid (0.01002 g as active ingredient) and bringing it to a volume of 10.0 mL with 0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1000 mg/L primary stock solution was typically prepared by placing 0.0251 g of anilino acid (0.0250 g as active ingredient) and bringing it to a volume of 25.0 mL with 0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1000 mg/L primary stock solution was typically prepared by placing 0.01004 g of diacid (0.01000 g as active ingredient) and bringing it to a volume of 10.0 mL with 0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1000 mg/L primary stock solution was typically prepared by placing 0.02581 g of 4-amino-3-chlorobenzoic acid (0.02504 g as active ingredient) and bringing it to a volume of 25.0 mL with 0.5% formic acid in acetonitrile. The primary stock solution was used to prepare mixed sub-stock solutions.

A 1000 mg/L primary stock solution was typically prepared by placing 0.0501 g of triphenyl phosphate (0.00500 g as active ingredient) and bringing it to a volume of 50.0 mL with 0.5% formic acid in acetonitrile. A 10.0 mg/L secondary stock solution was typically prepared by bringing 1.00 mL of the 1000 mg/L primary stock solution to a volume of 100 mL with 0.5% formic acid in acetonitrile. The 10.0 mg/L secondary stock solution was used as the internal standard in the foliage calibration standards.

A 20.0 mg/L mixed sub-stock solution was typically prepared by combining 0.200 mL of the 1000 mg/L tau-Fluvalinate, 3-PB acid, anilino acid, 4-amino-3-chlorobenzoic acid and diacid (diacid was excluded for the soil analysis) primary stock solutions with 0.200 mL of the 1060 mg/L haloanline primary stock solution and bringing it to a final volume of 10.0 mL with 0.5% formic acid in acetonitrile. A 2.00 mg/L mixed sub-stock solution was typically prepared by bringing 1.00 mL of the 20.0 mg/L mixed sub-stock solution to a volume of 10.0 mL with 0.5% formic acid in acetonitrile. The 20.0 mg/L mixed stock solution was used fortify the 10X LOQ level recovery samples and prepare the calibration standards. The 2.00 mg/L mixed stock solution was used fortify the LOQ level recovery samples and prepare the calibration standards.

All primary and secondary stock solutions were stored refrigerated in glass amber bottles fitted with Teflon[®]-lined caps. All mixed sub-stock solutions were prepared daily and discarded after use.

2.7 Reagent Solution and Mobile Phase Preparation

A 0.5% formic acid liquid reagent solution was typically prepared by mixing 5.00 mL of formic acid with 1.00 L of acetonitrile and mixed well.

A 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v) liquid reagent solution was typically prepared by mixing 450 mL of acetonitrile with 50.0 mL of purified reagent water and 2.50 mL of formic acid and mixed well.

A 0.1% acetic acid in acetonitrile mobile phase solution was typically prepared by mixing 2.00 mL of acetic acid with 2.00 L of acetonitrile. The solution was mixed well and degassed under vacuum with sonication.

A 0.1% acetic acid in purified reagent water mobile phase solution was typically prepared by mixing 2.00 mL of acetic acid with 2.00 L of purified reagent water. The solution was mixed well and degassed under vacuum with sonication.

A 30:30:40 acetonitrile:methanol:dimethyl sulfoxide (v:v:v) liquid reagent solution was typically prepared by mixing 1500 mL of acetonitrile with 1500 mL of methanol and 2000 mL of dimethyl sulfoxide. This solution was used as an autosampler wash on the LC/MS/MS instrument.

A 90:10 purified reagent water:acetonitrile (v:v) liquid reagent solution was typically prepared by mixing 900 mL of purified reagent water with 100 mL of acetonitrile. This solution was used as an autosampler purge rinse on the LC/MS/MS instrument.

2.8 Preparation of Calibration Standards

Matrix-matched calibration standards were prepared in raw control matrix extract (in a solution of 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v)) by fortifying with the 2.00 and 20.0 mg/L mixed sub-stock solutions of test substance and the 10.0 mg/L secondary stock solution of internal standard to yield concentrations of 10.0, 20.0, 40.0, 100 and 140 µg/L for soil sample analysis and 10.0, 20.0, 60.0, 100 and 140 µg/L for thatch and foliage sample analysis. Internal standard was ultimately only used during quantification of the foliage samples.

2.9 Sample Fortification and Preparation

All soil, thatch and foliage recovery samples (10.0 g dry weight for soil and thatch and 5.00 g for foliage) were weighed into individual 50-mL centrifuge tubes. Five replicates of each concentration were dosed with the appropriate test substance mixed sub-stock solutions at the LOQ (5.00, 10.0 and 20.0 ppb, respectively) and 10X LOQ (50.0, 100 and 200 ppb, respectively). The dosing procedure is detailed in the following table:

Sample ID	Matrix	Mixed Sub-Stock Concentration (mg/L)	Volume of Stock Solution (mL)	Matrix Weight (g)	Fortified Sample Concentration (ppb)
Reagent Blank	Soil	NA ^a	NA	NA	0.00
	Thatch	NA ^a	NA	NA	0.00
	Foliage	NA ^a	NA	NA	0.00
Control A & B	Soil	NA	NA	10.0	0.00
Control C & D	Thatch	NA	NA	10.0	0.00
Control E & F	Foliage	NA	NA	5.00	0.00
LOQ A, B, C, D & E	Soil	2.00	0.0250	10.0	5.00
LOQ F, G, H, I & J	Thatch	2.00	0.0500	10.0	10.0
LOQ K, L, M, N & O	Foliage	2.00	0.0500	5.00	20.0
10X LOQ A, B, C, D & E	Soil	20.0	0.0250	10.0	50.0
10X LOQ F, G, H, I & J	Thatch	20.0	0.0500	10.0	100
10X LOQ K, L, M, N & O	Foliage	20.0	0.0500	5.00	200

^a NA = Not Applicable

Two additional 5.00 or 10.0 g samples were prepared and left unfortified to serve as controls for each matrix. One additional sample was extracted using only extraction solvents to serve as the reagent blank.

2.10 Extraction Procedure

Following fortification, samples were allowed to sit for 30 minutes. Next, an aliquot of 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v) (5.00-mL for soil or 10.0-mL for thatch and foliage) was added to each reagent blank and recovery sample (10.0 g for soil (dry weight) and thatch or 5.00 g for foliage). The samples were then placed on an orbital shaker table for ten minutes at 250 rpm. After shaking, a 20- μ L aliquot of the 10.0 mg/L internal standard secondary stock solution was added to each sample. Samples were then centrifuged at 3000 rpm for ten minutes. If the supernatant was not sufficiently separated from the matrix, samples were centrifuged for an additional five minutes. An aliquot of the thatch and foliage

supernatant were centrifuged at 13,000 rpm for five minutes in low-binding centrifuge tubes prior to analysis to remove remaining particles. Samples and calibration standards were transferred to autosampler vials for LC/MS/MS and GC/MS analysis. The extraction and dilution procedure is summarized in the table below.

Sample ID	Matrix	Fortified Concentration (ppb)	Matrix Weight (g)	Final Volume ^a (mL)	Dilution Factor
Reagent Blank	Soil	0.00	NA ^b	5.00	1.00
	Thatch	0.00	NA	10.0	1.00
	Foliage	0.00	NA	10.0	1.00
Control A & B	Soil	0.00	10.0	5.00	0.500
Control C & D	Thatch	0.00	10.0	10.0	1.00
Control E & F	Foliage	0.00	5.00	10.0	2.00
LOQ A, B, C, D & E	Soil	5.00	10.0	5.00	0.500
LOQ F, G, H, I & J	Thatch	10.0	10.0	10.0	1.00
LOQ K, L, M, N & O	Foliage	20.0	5.00	10.0	2.00
10X LOQ A, B, C, D & E	Soil	50.0	10.0	5.00	0.500
10X LOQ F, G, H, I & J	Thatch	100	10.0	10.0	1.00
10X LOQ K, L, M, N & O	Foliage	200	5.00	10.0	2.00

^a Extracted with 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v).

^b NA = Not Applicable.

In addition to the fortified recovery samples, six aliquots of each matrix (20.0 g for soil and thatch, 10.0 g for foliage) were weighed into 50-mL centrifuge tubes and extracted with 90:10 acetonitrile:purified reagent water with 0.5% formic acid (v:v) (10 mL for soil, 20 mL for thatch and foliage). These samples were extracted in the same manner as the fortified recovery samples. The raw control extract generated by these samples were utilized to prepare matrix-matched standards for soil, thatch and foliage, respectively.

2.11 Analysis

2.11.1 Instrumental Conditions

The LC/MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column: Luna C-18 5 μ m, 3.0 \times 150 mm
 Column Oven Temp: 35 $^{\circ}$ C
 Flow Rate: 0.6 mL/min
 Injection Volume: 10.0 μ L
 Run Time: 32.00 minutes
 Retention Time: approximately 1.8 minutes for 4-amino-3-chlorobenzoic acid
 approximately 2.8 minutes for diacid
 approximately 5.1 minutes for 3-PB acid
 approximately 9.8 minutes for anilino acid
 approximately 10.8 minutes for triphenyl phosphate (IS)
 approximately 20.9 minutes for tau-Fluvalinate
 Mobile Phase A: 0.1% acetic acid in purified reagent water
 Mobile Phase B: 0.1% acetic acid in acetonitrile
 Autosampler Wash: 30:30:40 acetonitrile:methanol:dimethyl sulfoxide (v:v:v)
 Autosampler Purge: 90:10 purified reagent water: acetonitrile (v:v)
 Gradient Flow:

Time (minutes)	Solvent A (%)	Solvent B (%)
0.00	55.0	45.0
24.00	5.0	95.0
26.00	5.0	95.0
28.00	55.0	45.0
32.00	55.0	45.0

MS Conditions:

Instrument: AB Sciex API 5000 mass spectrometer equipped with an AB Sciex Turbo V ESI Ion Spray source
 Q1/Q3 mass: 170.00/126.00 amu (primary transition for 4-amino-3-chlorobenzoic acid)
 213.10/93.10 amu (primary transition for 3-PB acid)
 270.00/154.90 amu (primary transition for diacid)
 270.00/146.10 amu (confirmatory transition for diacid)
 294.10/145.10 amu (primary transition for anilino acid)
 294.10/127.20 amu (confirmatory transition for anilino acid)
 327.20/77.10 amu (primary transition for triphenyl phosphate)
 327.10/153.10 amu (confirmatory transition for triphenyl phosphate)
 503.20/180.90 amu (primary transition for tau-Fluvalinate)
 503.20/208.10 amu (confirmatory transition for tau-Fluvalinate)

Dwell time: 200 milliseconds
Scan type: MRM
Ion source: ESI
Source temperature: 600 °C
Resolution Q1/Q3: Unit/Unit
Curtain gas: 30.00
Collision gas: 10.00

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Instrument Parameter:	4-amino-3-chlorobenzoic acid	Diacid		Anilino acid		3-PB acid	Triphenyl phosphate (IS)		tau-Fluvalinate	
	Primary	Primary	Confirmatory	Primary	Confirmatory	Primary	Primary	Confirmatory	Primary	Confirmatory
Period (minutes)	0 - 2.14	2.14 - 10.30		2.14 - 10.30		2.14 - 10.30	10.30 - 15.55		15.55 - 32.00	
Ion Source Gas 1/Gas 2	80.00/80.00	30.00/50.00	30.00/50.00	30.00/50.00	30.00/50.00	30.00/50.00	50.00/50.00	50.00/50.00	80.00/80.00	80.00/80.00
Ionization Mode	negative	negative	negative	negative	negative	negative	positive	positive	positive	positive
Ion Spray Voltage	-3000.00	-1500.00	-1500.00	-1500.00	-1500.00	-1500.00	3500.00	3500.00	5500.00	5500.00
Declustering Potential	-80.00	-100.00	-100.00	-50.00	-110.00	-166.00	20.00	100.00	50.00	72.00
Entrance Potential	-10.00	-10.00	-10.00	-10.00	-10.00	-10.00	10.00	10.00	10.00	10.00
Collision Energy	-17.00	-16.00	-29.00	-30.00	-30.00	-30.00	39.00	30.00	44.00	19.00
Collision Cell Exit Potential	-15.00	-15.00	-15.00	-15.00	-35.00	-15.00	15.00	15.00	22.00	22.00

The GC/MS analysis was conducted utilizing the following instrumental conditions:

GC Parameters

Column: Agilent DB-5 MS,
 30 m × 0.25 mm (internal diameter) × 0.25 μm (film thickness)
 Temperature: 70 °C (initial) and held for 1.00 minute
 Ramps:

Rate (°C/minute)	Final Temperature (°C)	Hold Time (minutes)
5.00	200	5.00
40.00	300	10.00

Run time: 44.50 minutes
 Injection volume: 2.0 μL
 Carrier gas/flow: Helium, at a constant flow of 1.20 mL/minute
 Inlet mode: Pulsed splitless
 Pulse Pressure: 25.0 psi
 Purge time: on at 0.50 min at 50 mL/minute
 Inlet temperature: 250 °C
 Retention time:

Test Substance	Approximate Retention Times (minutes)
haloanline	9.8
triphenyl phosphate (IS)	34.5

MS Parameters

Solvent delay: 4.00 minutes
 Selected ion monitoring:

Ion (m/z)	Dwell (msec)	Comments
195.00	100	quantitation ion haloanline
326.10	100	quantitation ion triphenyl phosphate (IS)

Temperatures: MSD Transfer Line: 280 °C
 MS Quad: 150 °C
 MS Source: 230 °C

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11.3 Method Differences

No protocol or SOP deviations were generated during this validation. The following method deviations occurred during the study; however, these modifications did not generate any negative impacts on the study results:

- Wellmark International study number 4548 indicates to centrifuge the samples at 4000 rpm; however, this speed was not achievable with the available equipment. All samples were centrifuged at 3000 rpm.
- Wellmark International study number 4548 does not include a second centrifugation step if particles were present after extraction; however, thatch and foliage samples and calibration standards were centrifuged a second time at 13,000 rpm for five minutes prior to analysis to remove remaining particles.
- Wellmark International study number 4548 indicates to analyze primary and confirmation transitions for ACBA, diacid, 3-PB acid, RCAA, TPP and tau-Fluvalinate; however, only primary transitions were analyzed for ACBA and 3-PB acid due to sensitivity limitations of the LC/MS/MS used.
- Wellmark International study number 4548 utilizes an internal standard (TPP) during quantitation for the matrices of soil, thatch and foliage. During the ILV, internal standard quantitation was only utilized during foliage – soil and thatch utilized external standard calibration. The internal standard was not necessary for the soil and thatch matrices.

2.12 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the LOQ and 10X LOQ recovery samples. Recoveries of 70 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the LOQ and 10X LOQ recovery samples for each analyte. The retention time should have an RSD of less than or equal to 2%. The RSD of

the peak area based quantitation and of the recovery values should be less than or equal to 20%. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as tau-Fluvalinate and its metabolites which might interfere with the quantitation of analytes. Interferences with peak areas that are less than 50% at the limit of quantitation (LOQ) are not considered significant. Linearity of the method was determined by the correlation coefficient (r^2), y-intercept and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient (r) not less than 0.995. The precision of the method at the LOQ was reported in terms of the relative standard deviation or coefficient of variation of the observed recovery values.

2.13 Communications

Communications with the Sponsor Monitor, Method Developer and other pertinent Sponsor personnel occurred to discuss the test materials, method development testing and any applicable method modifications necessary prior to the start of the ILV testing. In addition, the use of internal standard, confirmation transitions and interpretation of study results upon completion of the ILV testing were discussed as well.

A complete list of communications is provided in [Appendix 3](#).

2.14 Time Required for Analysis

A normal batch of samples consists of a reagent blank, 10 fortified and 2 unfortified samples, 1 matrix-matched standard blank and 5 matrix-matched standards (19 samples total) for each matrix. A single analyst completed a set of 19 samples in one working day (8 hours) with LC/MS/MS and GC/MS analysis performed overnight.

3.0 Calculations

For the LC/MS/MS analysis, a calibration curve was constructed by plotting the analyte concentration ($\mu\text{g/L}$) in the calibration standards against the peak area (area ratio when internal

standard was used) of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the peak area (area ratio when internal standard was used) of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \quad y = ax^2 + bx + c$$

$$(2) \quad DC(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

$$(3) \quad A = DC \times DF$$

where:

- y = detector response (peak area or area ratio) for analyte
- a, b and c = regression constants
- DC (x) = detected concentration ($\mu\text{g/L}$) in the sample
- C = constant c minus the peak area or area ratio; $C = (c - y)$
- DF = dilution factor (the final sample volume divided by the original sample volume)
- A = concentration of the analyte in the original sample

For the GC/MS analysis, a calibration curve was constructed by plotting the analyte concentration ($\mu\text{g/L}$) of the calibration standards against the natural logarithm (\ln) of the peak area of the analyte in the calibration standards. The equation of the line (equation 4) was algebraically manipulated to give equation 5. The concentration of test substance in each recovery sample was calculated using the slope and intercept of the regression analysis, and the natural logarithm of the peak area and the dilution factor of the recovery sample. Equations 5, 6 and 7 were then used to calculate measured concentrations and analytical results.

$$(4) \ln y = m(\ln x) + b$$

$$(5) \ln x = \frac{(\ln y - b)}{m}$$

$$(6) DC(x) = \text{inverse}(\ln x)$$

$$(7) A = DC \times DF$$

where:

- $\ln x$ = natural logarithm of sample concentration
- $\ln y$ = natural logarithm of detector response
- m = slope from regression analysis
- b = y-intercept from regression analysis
- $DC(x)$ = detected concentration (mg/L) in the sample
- DF = dilution factor (final volume of the sample divided by the original sample volume)
- A = analytical result (mg/L)

The method limit of detection (LOD) was calculated by evaluating the signal-to-noise (S/N) ratio from samples of a known concentration (i.e. the lowest calibration standard) and blank samples (i.e. control samples) to establish the lowest level at which the analyte can reliably be detected. A S/N ratio of 3:1 was used to determine the LOD for each analyte and transition.