SUMMARY

An environmental chemistry method (ECM) was developed and successfully validated for the analysis of tau-fluvalinate and its metabolites (4-Amino-3-chlorobenzoic acid (ACBA), 2-(2-chloro-4-carboxyl)anilino-3-methylbutanoic acid (Diacid), 3-phenoxybenzoic acid (PBA), 2-(2-chloro-4-trifluoromethyl) anilino-3-methylbutanoic acid (RCAA), and 2-chloro-4-trifluoromethylaniline (haloaniline) in soil, thatch and foliage.

Extracted residue levels of tau-Fluvalinate, ACBA, Diacid, PBA, and RCAA were determined using LC-MS/MS and haloaniline was determined using GC/MS. PB aldehyde was found to be unstable in soil and thatch, degrading to PBA; therefore, analyses targeted PBA rather than PB aldehyde. Similarly, diacid was observed to degrade rapidly to ACBA in soil thus ACBA was quantified in soil analyses instead of diacid.

Linearity for the method was determined by preparing calibration curves of concentration against the instrument response for matrix-matched standards solutions within the range of 0.1–1.4 µg for thatch and foliage and 0.5–0.7 µg for soil. The regression coefficients (r2) ranged from 0.938 – 0.9999. The limit of quantitation (LOQ) for all test substances in soil was established at 5 ppb. For thatch and foliage, the LOQ was set at 10 ppb and 20 ppb, respectively, except for ACBA in foliage, where LOQ was determined to be 200 ppb.

Any potential interferences detected were not considered significant, being less than 50% of the LOQ. The calculated limit of detection (LOD) for the method for the test substances was about 3 µg/L in all matrices, except for ACBA in foliage, where LOD was 30 µg/L.

The developed method was also successfully validated by an independent laboratory (Smithers Viscient) as required by U.S. EPA Guideline Ecological Effects Test Guidelines OCSPP 850.6100.

In conclusion, the method for the Determination of tau-Fluvalinate and its Metabolites in Soils and Turf presented here was determined to be specific, precise, and accurate, and can be reliably used to quantify tau-Fluvalinate and metabolites ACBA, Diacid, PBA, RCAA and haloaniline in soil, thatch and foliage samples in field dissipation studies.

1.0 INTRODUCTION

The objective of this study was to develop an environmental chemistry method (ECM) for the analysis of tau-Fluvalinate and its metabolites in soil and turf (thatch and foliage) for a field dissipation study. The developed methods were also validated under GLP[2] by an independent laboratory as required by US Environmental Protection Agency (EPA) OCSPP 850.6100.[3]

A field dissipation study was required for the EPA 2012 Data Call-In for tau-fluvalinate.[4] The metabolites of tau-fluvalinate were selected based on the previous metabolic fate studies of tau-fluvalinate and the Registration Review -Preliminary Problem Formulation for the Ecological Risk and Drinking Water Exposure Assessment for tau-fluvalinate.[5-8] This method was developed to extract and quantitate each metabolite in soil, thatch, and foliage using LC-MS/MS for ACBA, diacid, PBA, RCAA, tau-fluvalinate and GC-MS for haloaniline.

2.0 MATERIALS AND METHODS

2.1 Test Substance and Internal Standard

2.1.1 Test Substances

Table 1 summarizes the test substances used in this study.

2.1.2 Internal Standard

Triphenyl phosphate (TPP) was used as internal standard. Below is a summary of the internal standard used.

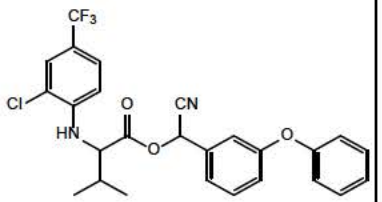
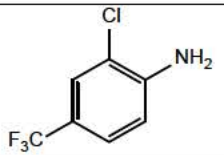
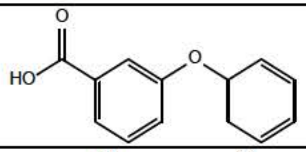
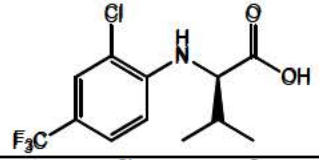
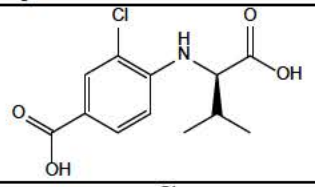
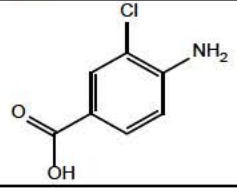
Name:	Triphenyl phosphate
Abbreviation:	TPP
CAS No.:	115-86-6
ID:	TDA08139
LOT:	EJ00TPH0014
Expiration Date:	07-31-2015
Provider:	United Phosphorus LTD

2.2 Reagents

The following reagents were used for this study.

	Reagent	**Purchased**	***Grade***	***ID #.***
1.	Acetonitrile:	Honeywell	*HPLC*	TDA8099
2.	Formic acid:	Macron,	*Reagent*	ENV06190
3.	Glacial acetic acid:	J. T Baker	*Reagent*	TDA 08083
4.	Purified water	Honeywell		TDA8149

Table 1. Test substances

Test Substance	CASRN	ABBREVIATION	EXACT MASS CHEM. FORMULA	STRUCTURE	LOT NO.	Exp. Date	Purity (%)
tau-Fluvalinate, (RS)-α-cyano-3-phenoxybenzyl-R-2-(2-chloro-4-trifluoromethyl)-anilino-3-butanoate	102851-06-9	TF	Mass: 502.1271 MF: $C_{26}H_{22}ClF_3N_2O_3$		ARS14-35-MIP2	07/31/2015	91.49
2-Chloro-4-trifluoromethylaniline	39885-50-2	Haloaniline	Mass: 195.0063 MF: $C_7H_5ClF_3N$		ARS14-57-HALO	08/31/2016	98.70
3-Phenoxybenzoic acid	3739-38-6	PBA	Mass: 214.0630 MF: $C_{13}H_{10}O_3$		ARS14-58-PBA	08/31/2016	99.40
2-(2-Chloro-4-trifluoromethyl)anilino-3-methylbutanoic acid	76769-07-8	RCAA (anilino acid)	Mass: 295.0587 MF: $C_{12}H_{13}ClF_3NO_2$		ARS14-56-RCAA	08/31/2016	99.57
2-(2-Chloro-4-carboxyl)anilino-3-methylbutanoic acid	85236-41-5	Diacid	Mass: 271.0611 MF: $C_{12}H_{14}ClNO_4$		ARS14-60-DIAC	08/31/2016	99.61
4-Amino-3-chlorobenzoic acid	2840-28-0	ACBA	Mass: 171.0087 MF: $C_7H_6ClNO_2$		ARS14-76-CABA	08/30/2016	97.0

2.3 Test System

The test system used for this study soil, foliage and thatch were collected from EPA Region 10 located in California. The test system was used to prepare matrix spikes and matrix-matched solutions for the method validation study.

TABLE 2. Test System

Area	EPA Region	ID No.	Crop	Soil Type
California	10	S-14-06976	Soil	Sandy Loam
California	10	S-14-06977	Foliage	Not Applicable
California	10	S-14-06978	Thatch	Sandy Loam

2.4 Equipment

1. Instrument: Agilent series 7890B gas chromatograph equipped with an Agilent series 5977A mass selective detector;
   Agilent series 1200 high performance Liquid chromatography system (HPLC) coupled to an Agilent G6410 triple quadrupole mass spectrometer operating in the multiple reaction monitoring (MRM) mode.
2. Balance: Mettler, AE163
   Mettler PE 1600
3. Centrifuge: Beckman Coulter Allegra X-22R
4. Laboratory equipment: 50 mL QuEChERS type centrifuge tubes (VWR, part # 82050-320)
   13 mm syringe filter with 0.45 µm PVDF membrane (Pall, part # 4452T)
   Hamilton® gas tight syringes with appropriate microliter delivery volumes.
   Class A volumetric glassware

2.5 Preparation of Stock Solutions

A tau-fluvalinate stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting solution contained 1023 mg/L tau-fluvalinate.

A PBA stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting solution contained 1033 mg/L PBA.

A haloaniline stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting solution contained 1334 mg/L haloaniline.

A RCAA stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting stock solution was 1042 mg/L RCAA.

A diacid stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting stock solution was 1034 mg/L diacid.

An ACBA stock solution was prepared in acetonitrile containing 0.5% formic acid. The resulting solution contained 1036 mg/L ACBA

Although PB aldehyde was ultimately found to be unstable and not able to be quantitatively recovered from sample matrices, a stock solution was initially prepared and included in the mixed-spiking solution. The PB aldehyde stock solution was prepared in acetonitrile containing 0.5% formic acid resulting in a solution containing 1563 mg/L PB aldehyde.

A stock solution of TPP was prepared in acetonitrile containing 0.5% formic acid to give concentration of 1023 µg/mL which was further diluted to 10.23 µg/mL. Although included in the stock standard, use of the ISTD was found unnecessary due to its inconsistent response and minimal moisture in the samples; external standards were used for quantification.

2.6 Preparation of Extraction Solution

Extraction solution (acetonitrile/water 90/10 with 0.5% formic acid) was prepared by mixing 100 mL of water, 900 mL acetonitrile, and 5 mL of formic acid.

2.7 Preparation of Sub-Stock Solutions

2.7.1 Mixed-Spiking Solution (A)

Mixed-Spiking Solution (A) was prepared by pipetting an appropriate amount of each stock solution (1023 mg/L tau-fluvalinate, 1033 mg/L PBA, 1334 mg/L haloaniline, 1042 mg/L RCAA, and 1034 mg/L diacid, 1563 mg/L PB aldehyde) into the same 50 mL volumetric flask and brought to 50 mL volume with acetonitrile containing 0.5% formic acid. The final concentration of each analyte was about 20 µg/mL.

2.7.2 Mixed-Spiking Solution (B)

Mixed-Spiking Solution (B) was prepared by pipetting 5 mL of the mixed spiking solution (A) into 50 mL volumetric flask and diluting it with acetonitrile containing 0.5% formic acid. The final concentration of each analyte was about 2 µg/mL.

2.7.3 ACBA

The ACBA stock solution was further diluted with acetonitrile containing 0.5% formic acid to prepare 10× LOQ spiking solution and LOQ spiking solution of 20.72 and 2.072 µg/mL, respectively. ACBA was not included in Mixed-Spiking Solution (A)/(B) to avoid quantification of ACBA, as diacid can convert to ACBA.

2.8 Soil Analysis

2.8.1 Matrix-Matched Calibration Solutions for Soil

Placebo soil extract was prepared by weighing 20 g of placebo soil in QuEChERS type tube, then 10 mL of extraction solution was added. The solution was shaken and centrifuged at 4000 rpm for 10 minutes. Aliquots of 5 mL from the supernatant was measured out into another QuEChERS type tube to prepare standard calibration solutions. The procedure was repeated six times to prepare six replicates of the placebo soil extract. The first tube, 5 mL of aliquot, was spiked with 25 µL of 2 µg/mL of Mixed-Spike solution (B). Each individual 5 mL of soil-extract aliquot was spiked with 5 µL, 10 µL, 25 µL, and 35 µL of Mixed-Spike Solution (A), respectively. One tube was left unspiked to be used as a control. After spiking each with 20 µL of ISTD, standard solutions were filtered using a 13 mm syringe filter with a pore size of 0.45 µm prior to LC-MS/MS and GC/MS analyses. Each calibration standard was run twice.

The prepared matrix-matched standard solutions were used to establish a calibration curve. The concentration for each analyte is summarized in Table 3.

2.8.2 Matrix-Matched ACBA Calibration Solution for Soil

Placebo soil extract was prepared by weighing 20 g of placebo soil in QuEChERS type tube, then 10 mL of extraction solution was added. The solution is shaken and centrifuged at 4000 rpm for 10 minutes. Aliquots of 5 mL from the supernatant was measured out into another QuEChERS type tube to prepare standard calibration solutions. The procedure was repeated six times to prepare six replicates of the placebo soil extract. The first tube, 5 mL of aliquot, was spiked with 25 µL of ACBA LOQ spiking solution. Each 5 mL of soil extract was spiked with 5 µL, 10 µL, 25 µL, and 35 µL of ACBA 10×LOQ spiking solution, respectively. One tube was left unspiked to be used as a control. Each standard level was spiked with 20 µL of 10.2 µg/mL TPP as an internal standard. Each standard solution was filtered using a 13 mm syringe filter with a pore size of 0.45 µm prior to LC-MS/MS and was run twice. The prepared matrix-matched standard solutions were used to establish a calibration curve.

2.8.3 Soil Sample Fortification

For recovery analysis, 10 g of soil sample was weighed in a 50 mL QuEChERS type centrifuge tube. This procedure was repeated twelve times. The first five samples were each spiked with 25 µL of Mixed-Spiking Solution (B) to reach LOQ level (5 replicates) and the other five replicates were each spiked with 25 µL of Mixed Spiking Solution (A) to reach 10× LOQ level. The remaining two sets of 10 g of soil samples were left unspiked to be used as controls. The spiked samples were left for one hour to equilibrate before 5 mL of extraction solution and 20 µL ISTD were added. The sample tubes were then mixed thoroughly and centrifuged for 10 minutes at 4000 rpm. The supernatant was filtered using a syringe filter with a pore size of 0.45 µm. The filtrate was assayed twice using LC-MS/MS for Diacid, PBA, RCAA, and tau-fluvalinate against matrix-matched calibration solution under MRM scan and using GC/MS for haloaniline.

PB aldehyde was found to be unstable in soil and thatch, degrading to PBA; therefore, analyses targeted PBA rather than PB aldehyde. Similarly, diacid was observed to degrade rapidly to ACBA in soil thus ACBA was quantified in soil analyses instead of diacid.

Table 3. Concentration of each analyte in soil matrix-matched calibration standard solutions				
Analyte	**Concentration in Spiking Solution (µg/mL)**	**Calibration Standard Solution**	**Spiking Volume (µL)**	**Concentration (µg)**
Tau-Fluvalinate	2.05	STD-1	25.0	0.051
	20.5	STD-2	5.0	0.102
		STD-3	10.0	0.205
		STD-4	25.0	0.512
		STD-5	35.0	0.716
ACBA	2.07	STD-1	25	0.052
	20.7	STD-2	5.0	0.104
		STD-3	10.0	0.207
		STD-4	25.0	0.518
		STD-5	35.0	0.725
DIACID	2.07	STD-1	25.0	0.052
	20.7	STD-2	5.0	0.103
		STD-3	10.0	0.207
		STD-4	25.0	0.517
		STD-5	35.0	0.724
PBA	2.07	STD-1	25.0	0.052
	20.7	STD-2	5.0	0.103
		STD-3	10.0	0.207
		STD-4	25.0	0.517
		STD-5	35.0	0.723
PB ALDEHYDE	2.03	STD-1	25.0	0.051
	20.3	STD-2	5.0	0.102
		STD-3	10.0	0.203
		STD-4	25.0	0.508
		STD-5	35.0	0.711
RCAA	2.08	STD-1	25.0	0.052
	20.8	STD-2	5.0	0.104
		STD-3	10.0	0.208
		STD-4	25.0	0.521
		STD-5	35.0	0.729
HALOANILINE	2.00	STD-1	25.0	0.050
	20.0	STD-2	5.0	0.100
		STD-3	10.0	0.200
		STD-4	25.0	0.500
		STD-5	70.0	0.700

2.9 Thatch Analysis

2.9.1 Matrix-Matched Calibration Solutions for Thatch

Placebo thatch extract was prepared by weighing 20 g of placebo thatch in QuEChERS type tube, followed by adding 20 mL of extraction solvent. The mixture is shaken and centrifuged at 4000 rpm for 10 minutes. The upper 10 mL of the extracted solution was transferred to another QuEChERS type tube followed by spiking with 50 µL of 2 µg/mL of mixed spike solution (B). Likewise, the above placebo thatch extraction procedure was repeated five times to collect four additional 10 mL placebo thatch extracts. Each individual thatch extract was then spiked respectively with 10 µL, 30 µL, 50 µL, and 70 µL of Mixed Spike Solution (A). One thatch extract was left unspiked to be used as control. After spiking each standard with 20 µL of ISTD, standard solutions were filtered using a syringe filter with a pore size of 0.45 µm prior to LC-MS/MS and GC/MS analyses. Each standard was run twice. The prepared matrix-matched standard solutions were used to establish a calibration curve.

2.9.2 Matrix-Matched ACBA Calibration Solutions for Thatch

Placebo thatch extract was prepared by weighing 10 g of placebo thatch in QuEChERS type tube, followed by adding 20 mL of extraction solvent. The mixture was shaken and centrifuged at 4000 rpm for 10 minutes. The upper 10 mL of the extracted solution was transferred to another QuEChERS type tube followed by spiking with 50 µL of 2 µg/mL of ACBA (LOQ standard solution). Likewise, the above placebo thatch extraction procedure was repeated five times to collect five additional 10 mL placebo thatch extracts. Each individual thatch extract was then spiked respectively with 10 µL, 30 µL, 50 µL, and 70 µL of 20 µg/mL ACBA (10× LOQ standard solution). One tube was left unspiked to be used as a control. After spiking each standard with 20 µL of ISTD, standard solutions were filtered using a 13 mm syringe filter with a pore size of 0.45 µm prior to LC-MS/MS analysis. Each standard was run twice. The prepared matrix-matched standard solutions were used to establish a calibration curve. The concentration for each analyte is summarized in Table 4.

Table 4. Concentration of each analyte in thatch and foliage matrix-matched calibration standard solutions

Analyte	Concentration in Spiking Solution (µg/mL)	Calibration Standard Solution	Spiking Volume (µL)	Concentration (µg)
Tau-Fluvalinate	2.05	STD-1	50.0	0.102
	20.5	STD-2	10.0	0.205
		STD-3	30.0	0.614
		STD-4	50.0	1.023
		STD-5	70.0	1.432
ACBA	2.07	STD-1	50.0	0.104
	20.7	STD-2	10.0	0.207
		STD-3	30.0	0.622
		STD-4	50.0	1.036
		STD-5	70.0	1.450
DIACID	2.07	STD-1	50.0	0.103
	20.7	STD-2	10.0	0.207
		STD-3	30.0	0.620
		STD-4	50.0	1.034
		STD-5	70.0	1.448
PBA	2.07	STD-1	50.0	0.103
	20.7	STD-2	10.0	0.207
		STD-3	30.0	0.620
		STD-4	50.0	1.033
		STD-5	70.0	1.446
PB ALDEHYDE	2.03	STD-1	50.0	0.102
	20.3	STD-2	10.0	0.203
		STD-3	30.0	0.610
		STD-4	50.0	1.016
		STD-5	70.0	1.422
RCAA	2.08	STD-1	50.0	0.104
	20.8	STD-2	10.0	0.208
		STD-3	30.0	0.625
		STD-4	50.0	1.042
		STD-5	70.0	1.459
HALOANILINE	2.00	STD-1	50.0	0.100
	20.0	STD-2	10.0	0.200
		STD-3	30.0	0.600
		STD-4	50.0	1.001
		STD-5	70.0	1.401

2.9.3 Thatch Sample Fortification

Ten grams of thatch was weighed into 50 mL QuEChERS type tube was spiked with 50 µL of 2 µg/mL mixed spiking solution (B) to reach LOQ levels (5 replicates) another five sets were spiked with 50 µL of 20 µg/mL mixed spiking solution (A) to reach 10 × LOQ level. The samples were allowed to sit for 30 minutes to 1 hour before 10 mL of extraction solution was added. The samples were spiked with 20 µL of ISTD and centrifuged for 10 minutes at 4000 rpm. After filtering the samples using 13 mm syringe filter with a pore size of 0.45 µm, the filtered extract was analyzed twice using LC-MS/MS under MRM scan for Diacid, PBA, PB aldehyde, RCAA, and tau-fluvalinate against matrix matched calibration solution. The same solution was assayed twice using GC/MS for haloaniline. ACBA was fortified separately in exact same way as the mixed standards to prepare two sets of five replicate samples at LOQ and 10× LOQ. The prepared samples were analyzed as duplicates using LC-MS/MS.

2.10 Foliage Analysis

2.10.1 Matrix-Matched Calibration Solutions for Foliage

Placebo foliage extract was prepared by weighing 10 g of placebo foliage in QuEChERS type tube, followed by addition of 20 mL of extraction solvent. The mixture was centrifuged at 4000 rpm for 10 minutes. An additional five replicates were prepared the same way. Aliquots of 10 mL were measured out into centrifuge tubes. The first 10 mL extract was spiked with 50 µL of 2 µg/mL of mixed-spike solution (B). The remaining individual 10 mL aliquots of foliage extract were spiked respectively with 10 µL, 30 µL, 50 µL, and 70 µL of mixed-spiking solution (A). One 10 mL of foliage extract was left unspiked to be used as control. After addition of 20 µL of ISTD, standard solutions were filtered using a 13 mm syringe filter with a pore size of 0.45 µm prior to LC-MS/MS analysis. The concentration for each analyte is summarized in Table 4.

2.10.2 Matrix-Matched ACBA Calibration Solutions for Foliage

Placebo foliage extract was prepared by weighing 10 g of placebo thatch in QuEChERS type tube, followed by adding 20 mL of extraction solvent. The mixture is shaken and centrifuged at 4000 rpm for 10 minutes. The upper 10 mL of the extracted solution was transferred to another QuEChERS type tube followed by spiking with 50 µL of 2 µg/mL of ACBA (LOQ standard solution). Likewise, the above placebo foliage extraction procedure is repeated five times to collect five additional 10 mL placebo thatch extracts. Each individual thatch extract was then spiked respectively with 10 µL, 30 µL, 50 µL, and 70 µL of 20 µg/mL ACBA (10× LOQ standard solution). One 10 mL extract was left unspiked to be used as a control. After spiking each with 20 µL of ISTD, the standard solutions were filtered using a 13 mm syringe filter with a pore size of 0.45 µm prior to LC-MS/MS analysis. Each calibration standard was run twice. The prepared matrix-matched standard solutions are used to establish a calibration curve.

2.10.3 Foliage Sample Fortification

Twelve replicates of five-gram samples of foliage were weighed into 50 mL QuEChERS type tube. The first five replicates were spiked with 50 µL of 2 µg/mL mixed-spiking solution (B) to reach LOQ levels (5 replicates) and other five replicates were spiked with 50 µL of 20 µg/mL mixed-spiking solution (A) to reach 10× LOQ level. After the samples were let to sit for 1 hour, 10 mL of extraction solvent (acetonitrile/water: 90/10 containing 0.5% of formic acid) was added. After addition of 20 µL of ISTD the samples were centrifuged for 10 minutes at 4000 rpm. The upper layer was filtered using a syringe filter with a pore size of 0.45 µm prior to LC-MS/MS and GC/MS. All samples were run as duplicate.

ACBA was fortified separately in exact same way as the mixed standards to prepare two sets of five replicate samples at LOQ and 10× LOQ.

2.11 Analysis

2.11.1 LC-MS/MS Parameters

Chromatography Parameters:

Analytical Column:	Phenomenex Luna C18 (2), 150 x 3.0 mm ID, 5 µm
Flow Rate:	0.6 mL/min
Column Oven Temperature:	35°C
Injection Volume:	30 µL
Run Time:	32 minutes
Stop Time:	28 minutes
Post time:	4 minutes
Retention Time:	~1.9 minutes, ACBA
	~3.0 minutes, Diacid
	~5.9 minutes, PBA
	~9.2 minutes, PB Aldehyde
	~11.2 minutes, RCAA
	~12.3 minutes, TPP
	~22.3 minutes, tau-Fluvalinate
Mobile Phase A:	Water with 0.1% acetic acid in
Mobile Phase B:	Acetonitrile with 0.1% acetic acid
Gradient Flow*:*	

Table 5. LC gradient

Time (minutes)	***Mobile A (%)***	***Mobile B (%)***	
0.00	55	45	
24.00	5	95	
26.00	5	95	
28.00	55	45	
32.00	55	45	*Post run*

Mass Spectrometry parameters:

Ionization mode:	ESI
Scan type:	Multiple Reaction Monitoring (MRM)
Sheath gas temperature:	350°C
Sheath gas flow:	10 L/min (nitrogen)
Nebulizer:	40 psi
Capillary voltage:	3000 - 6000 V (Table 6 for each test substances)
Collision energy:	5 - 30 V (see Table 7 for each test substances)
Dwell time:	200 msec

Table 6. MRM transition parameters for each analyte.

Name	Time Seg.	Time (min)	Pre. Ion	Quant. Ion	CE for Quant. (V)	Confirm. Ion	CE for Confirm (V)	Dwell (msec)	Frag. (V)	Delta EMV	Cap. (V)	ESI Polarity
ACBA*	2*	1.0-2.4	170	126.0	8	NA[a]	8	200	100	600	3000	Neg.
Diacid	2	2.4-4.5	270	154.9	5	146.1	5	200	100	600	1500	Neg.
PBA	3	4.8-8.0	213.1	93.1	12	169.1	5	200	70	600	1500	Neg.
PB aldehyde	4	8.0-10.0	199.1	171.1	7	153.3	7	200	150	600	5000	Pos.
RCAA	6	10.0-11.9	294.1	145.1	18	127.2	18	200	110	600	1500	Neg.
TPP	7	11.9-20.0	327.2	77.1	40	153.1	30	200	170	600	3500	Pos.
tau-Fluvalinate	8	20.0-28.0	503.2	180.9	30	208.1	5	200	100	600	5500	Pos.

*Run separate

[a] Not applicable. No additional fragment ion was detected for this compound to be used as confirmation ion.

2.11.2 GC/MS Parameters

Chromatography Parameters

Column: DB-5 MS, 30 m × 0.25 mm ID × 0.25 µm film thickness

Oven Temperature: Summarized in table below.

Table 7. GC oven temperature program

Rate (°C/minute)	*Final Temperature (°C)*	*Hold Time (minutes)*
--	70	1
5	200	5
40	300	10

Run time:	44.5 minutes
Injection volume:	2.0 µL
Carrier gas:	Helium
Carrier gas flow rate:	1.2 mL/min under constant flow
Injection Mode:	Pulsed splitless
Pulse Pressure:	25 psi
Purge Time:	on at 0.5 min at 50 mL/min
Inlet Temperature:	250 °C
Retention Time	~11.7 minutes, haloaniline

MS Parameters:

Solvent delay:	10 minutes
Scan Type:	Selected ion monitoring (SIM) ion (*m/z*) 195
Dwell time:	100 ms
Temperatures:	MSD Transfer Line: 280 °C
	MS source: 230 °C
	MS Quad: 150 °C

2.11.3 Statistical Analysis of Data

The following commercial software programs were used in this study. Agilent MassHunter® Ver B.05 was used for data acquisition and for chromatographic area integration. In addition, Agilent MassHunter® Quantitative Ver B.07.01 was used for data acquisition for chromatographic peak area integration and for quantitative analyte determination and recovery calculations. Microsoft® Excel 2013/2016 was used for calibration curves plotting, concentration calculations, and statistical calculations.

3.0 CALCULATIONS

For the LC-MS/MS analysis of samples, the concentration of the spiked amount of test substance (µg) was calculated by multiplying the volume of the spiking solution (L) by the concentration of individual test substance in the mixed-spiking solution (µg/L). This concentration was used to construct the calibration curve by plotting against the instrument response (peak area of analyte) for each spiked level. Agilent Quantitative MassHunter Ver B.07.01 was used for peak area integration and quantitative determination of the analytes. Quadratic fit was used to fit the calibration curve (Equation 1). The detected concentration of test substance (x) in each recovery sample was then calculated by the software using Equation 2. The amount of the test substance in µg/g was finally calculated by dividing x by the sample weight (w) (Equation 3). Percent recovery is calculated using Equation 4.

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

$$2.\quad x\,(\mu g) = \frac{-b+\sqrt{b^2-4aC}}{2a}$$

$$3.\quad A = \frac{x}{y}$$

$$4.\quad \%\,recovery = \frac{concentration\ of\ analyte\ (\mu g)}{concentration\ of\ fortified\ standard\ (\mu g)}\ x\ 100\%$$

where

	y	=	Detector response (peak area) for analyte
	a, b and c	=	Regression constants
	x	=	Detected concentration in sample in µg
	w	=	*sample weight (g)*
	C	=	c - y
	A	=	final concentration in (µg/g)

For the GC/MS analysis of haloaniline, the calibration curve was constructed by plotting analyte concentration (ng/mL) of the calibration standards against the peak area of the analyte in the calibration standards. A linear regression (Equation 5) was statistically determined using Excel (Microsoft Office 2016). The detected concentration (x) of test substance in each recovery sample was calculated using the slope and intercept of the regression analysis (Equation 5). Percent recovery is calculated using Equation 7.

$$5.\quad y = ax + b$$

$$6.\quad x\,(ng/mL) = (y - b)/a$$

7. $\%\, recovery = \frac{concentration\ of\ analyte\ (ng/mL)}{concentration\ of\ fortified\ standard\ (ng/mL)}\ x\ 100\%$

Where

y	=	Detector response (peak area) for analyte
a	=	Slope
b	=	Intercept
x	=	Detected concentration in sample in ng/mL

4.0 PROTOCOL AMENDMENT AND DEVIATION

One amendment and one deviation are attached for reference in Appendix 1.

PROTOCOL AMENDEMENT AND DEVIATION

PROTOCOL AMENDMENT

Study Title: Analytical Method Validation for the Determination of tau-Fluvalinate and its Metabolites in Soils and Turf
Wellmark International Study No.: 4548

Amendment No.: 1

DESCRIPTION OF AMENDMENT: Metabolites 3-phenoxybenzaldehyde (PBAL) and 3-phenoxybenzaldehyde cyanohydrin were not analyzed as part of the method validation. Also, Diacid was not analyzed in the soil matrix.

Reason for Change: Both the 3-phenoxybenzaldehyde (PBAL) and 3-phenoxybenzaldehyde cyanohydrin were unstable and could not be quantitatively recovered from the sample matrices. The diacid was not analyzed in the soil matrix due to rapid degradation. It was analyzed in the thatch and foliage.

Impact of Change on Study:

No impact is expected due to this change.

Approved by:
Study Director:

Alice Welch D. Chem.
Study Director
Wellmark International

Aug 24, 2017
Date

Sponsor Study Monitor:

Jinren Ko, Ph.D.
Sponsor Representative
Wellmark International

Aug 24, 2017
Date

PROTOCOL DEVIATION

Study Title: Analytical Method Validation for the Determination of tau-Fluvalinate and its Metabolites in Soils and Turf

Wellmark International Study No.: 4548

Deviation No.: 1

DESCRIPTION OF DEVIATION: The ACBA metabolite LOQ in foliage was changed from 20 ppb to 200 ppb.

Reason for Deviation:

Foliage extract has a complex matrix that interfere significantly with the detection of ACBA. Depending on the complexity of this matrix extract and sensitivity of the mass spectrometry, the detection limit of ACBA can be varied. Hence to increase the confidence of ACBA quantification, the LOQ of ACBA was raised to 200 ppb. In addition, since the detection of ACBA is dependent on the complexity of the foliage matrices, it is highly recommended to include 20 ppb standard to the matrix-matched calibration. This will to ensure when matrix complexity permits, ACBA can be detected and quantified at or higher than 20 ppb.

Impact of Change on Study:

Raising the LOQ level will increase the confidence of detection of ACBA. In addition, since 20 ppb of ACBA will be included to the matrix-matched calibration solution, when matrix complexity permits, ACBA at or higher than 20 ppb in field samples will be detected and quantified. Hence, this change will only affect the quantification of ACBA in highly complex matrices only.

Approved by:
Study Director:

MAR 0 7 2018

Alice Welch, D.Chem.
Study Director
Wellmark International

APPENDIX 3

SUMMARY OF TAU-FLUVALINATE PHYSIOCHEMICAL PROPERTIES

Summary of tau-Fluvalinate Physiochemical Properties		
Parameter	**Value**	**Reference/Comments**
Chemical Classification	Pyrethroid	The Pesticide Manual, 2006
Pesticide Classification	Sodium Channel	
Molecular Weight	502.9 g/mol	The Pesticide Manual, 2006
Water Solubility	1.03 ppb (pH 7, 20°C)	The Pesticide Manual, 2006
Vapor Pressure	$9x10^{-8}$ mPa (20°C)	The Pesticide Manual, 2006
Henry's Law Constant	$4.04x10^{-5}$ Pa m3 mol^{-1} (calc)	The Pesticide Manual, 2006
Octanol Water Partition, K_{OW}	$>10^6$	W.I. Study No.: 2516
Log K_{OW}	4.26 (25°C)	The Pesticide Manual, 2006
Dissociation Constant pKa	Non-ionized	Metabolic Pathways of Agrochemicals
Melting Point	164°C/0.07 mmHg (tech.)	The Pesticide Manual, 2006
pH	5.3 (25°C)	C.C.L. Study No.: 1820-56
Density	1.262 (25°C)	The Pesticide Manual, 2006

The Pesticide Manual, 14th ed.; 2006, p. 520, Editor, C.D. Tomlin.

Wellmark International Study No.: 2516; Physical and Chemical Properties Tests for tau-Fluvalinate.

Metabolic Pathways of Agrochemicals: Part II, Pesticides and Fungicides, 1999, p. 670,
Editors, T. Roberts and D. Hutson.

Case Consulting Laboratories, Inc., Study No.: M1820.56, Physical and Chemical Characteristics of Tau-Fluvalinate Technical: Color, Physical State, Odor, Oxidation/Reduction, Flammability, Explodability, pH, Viscosity and Relative Density, 2005.