T I T L E

Independent Laboratory Validation of Analytical Methods for the Determination of Fluthiacet-methyl and Seven Metabolites in Soil and Water

TEST GUIDELINE

U.S. EPA Test Guideline OCSPP 850.6100

2.0 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of Fluthiacet-methyl (CGA-248757) and seven metabolites (CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059) in pond water and soil by Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS). The analysis of the reference/test substances was based on the analytical methods described in the study reports "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 in Water by High Performance Liquid Chromatography with Mass Spectrometric Detection" (Reference 1) and "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 in Soil by High Performance Liquid Chromatography with Mass Spectrometric Detection" (Reference 2) provided by the Sponsor.

This study was designed to satisfy US EPA Test Guideline requirements described in OCSPP 850.6100. The study was initiated on January 21, 2016. The experimental work was conducted from February 4 to September 17, 2016, at EAG Laboratories-Hercules, 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol (Appendix A) and according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160.

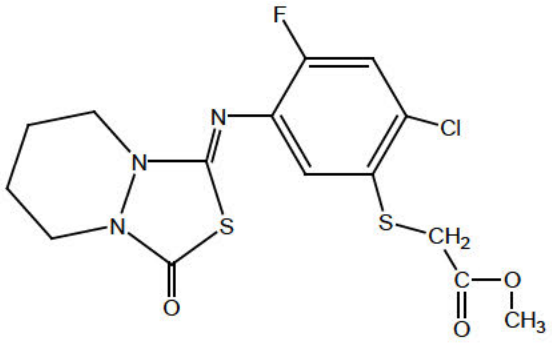
3.0 MATERIALS AND METHODS

3.1 Reference/Test Substances

Fluthiacet-methyl (CGA-248757) and metabolites CGA-300402, GA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 test/reference substances were provided by the Sponsor on January 21, 2016. Upon receipt at EAG Laboratories-Hercules, the test/reference substances were assigned the PTRL inventory numbers 2812W-001 through 2812W-008. All reference/test substances except for Fluthiacet-methyl were maintained frozen (typically < -4 °C) when not in use. Fluthiacet-methyl was maintained at room temperature when not in use.

Fluthiacet-methyl (CGA-248757)

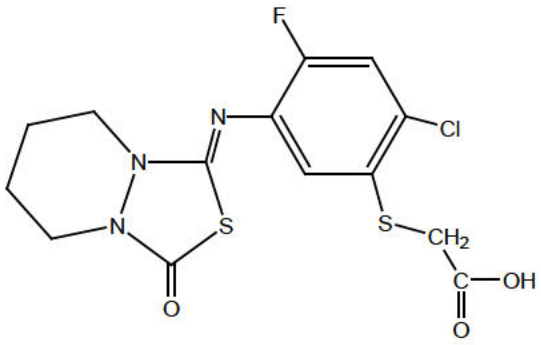
Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]thio]-methyl ester
CAS number:	117337-19-6
Molecular Formula:	$C_{15}H_{15}ClFN_3O_3S_2$
Molecular Weight:	403.88 g/mole
Structure:	



CGA-300402

Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]thio]-
CAS number:	149253-65-6
Molecular Formula:	$C_{14}H_{13}ClFN_3O_3S_2$
Molecular Weight:	389.85 g/mole

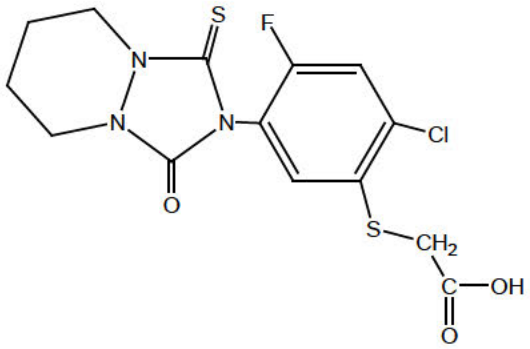
Structure:



CGA-300403

Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1-oxo-3-thioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2(3H)-yl)phenyl]thio]-
CAS number:	not available
Molecular Formula:	$C_{14}H_{13}ClFN_3O_3S_2$
Molecular Weight:	389.85 g/mole

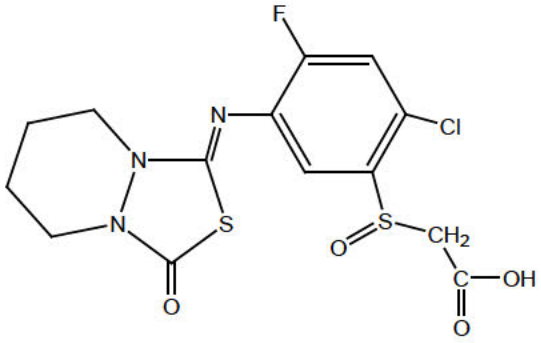
Structure:



CGA-300404

Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]sulfinyl]-
CAS number:	not available
Molecular Formula:	$C_{14}H_{13}ClFN_3O_4S_2$
Molecular Weight:	405.85 g/mole

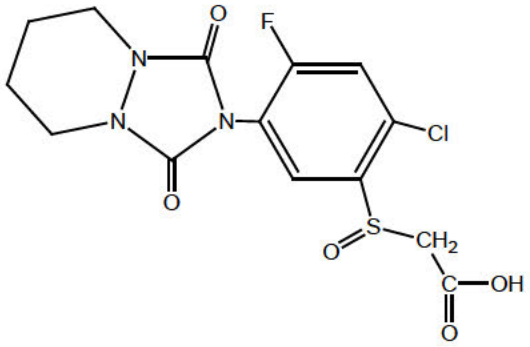
Structure:



CGA-327067

Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1,3-dioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2-(3H)-yl)phenyl]sulfinyl]-
CAS number:	not available
Molecular Formula:	$C_{14}H_{13}ClFN_3O_5S$
Molecular Weight:	389.79 g/mole

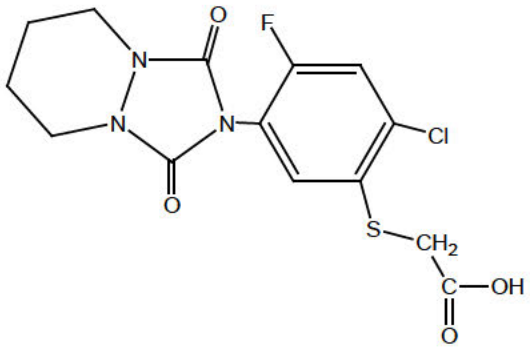
Structure:



CGA-327066

Chemical Name:	acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1,3-dioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2-(3H)-yl)phenyl]thio]-
CAS number:	not available
Molecular Formula:	$C_{14}H_{13}ClFN_3O_4S$
Molecular Weight:	373.79 g/mole

Structure:



CGA-330057

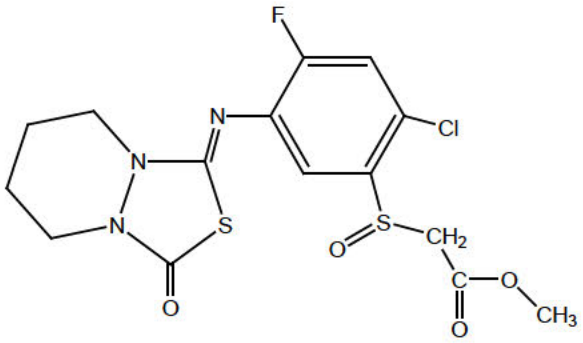
Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H-3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]sulfinyl]-,methyl ester

CAS number: not available

Molecular Formula: $C_{15}H_{15}ClFN_3O_4S_2$

Molecular Weight: 419.88 g/mole

Structure:



CGA-330059

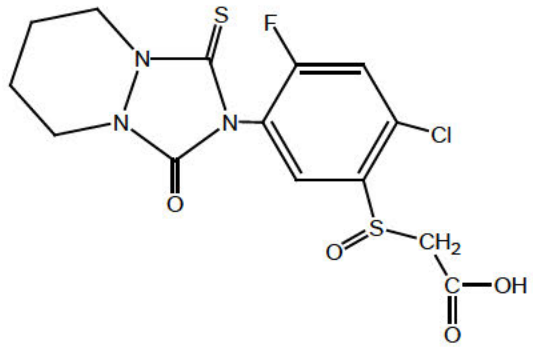
Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1-oxo-3-thioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2(3H)-yl)phenyl]sulfinyl]-

CAS number: not available

Molecular Formula: $C_{14}H_{13}ClFN_3O_4S_2$

Molecular Weight: 405.85 g/mole

Structure:



EAG Laboratories-Hercules No.	Analyte	Lot no.	Purity %	expiration date
2812W-001	Fluthiacet-methyl (CGA-248757)	PL07-0734/G26-54	99.3	February 28, 2017
2812W-002	CGA-300402	FM-19	99.2	February 28, 2019
2812W-003	CGA-300403	FM-20	98.3	June 30, 2019
2812W-004	CGA-300404	FM-6	93.7	April 30, 2017
2812W-005	CGA-327066	FM-22	95.4	June 30, 2017
2812W-006	CGA-327067	FM-23	92.3	June 30, 2017
2812W-007	CGA-330057	FM-9	99.6	June 30, 2017
2812W-008	CGA-330059	FM-21	96.3	June 30, 2017

3.2 Reagents

Acetonitrile and methanol were obtained from Burdick & Jackson; ammonium acetate and acetic acid were obtained from Fisher Scientific. All water used was HPLC grade or purified with a Thermo Scientific Barnstead Smart2pure system).

Sample diluent: 10:90 (v:v) ACN: 0.05 M ammonium acetate pH =5

3.2.1 0.05 M Ammonium Acetate in Water pH = 5

An aliquot of ammonium acetate (3.85 g) was weighed and added into 1 L purified water. An aliquot of acetic acid (1 mL) was added into the solution and mixed on a stir plate. The pH of final solution was verified (pH = 5) with a pH indicator paper. Other volumes were prepared during the study but same ratio of reagents was maintained.

3.2.2 43.5% (w/w) Ammonium acetate/ water

An aliquot of ammonium acetate (38.5 g) was weighed and added into 50 mL purified water.

3.3 Equipment/Materials List

Laboratory Balances
Plastic disposable centrifuge bottles (250 mL capacity)
Sieve 200 µm
Whatman pH indicator paper
Wrist-Action shaker
Rotary evaporators with water bath
Thermometers
Agilent Bond Elut SAX SPE cartridges (1 g/6 mL)
Agilent Bond Nexus SPE cartridges (0.5 g/ 12 mL)
Plastic reservoirs (75 mL capacity)
Adjustable volume pipetors with plastic disposable tips
Sonicator
Vortex mixer
Stir plate
Glassware:

- Graduated cylinders
- Beakers
- Weighing boats
- Hamilton glass precision syringes
- Round bottom flasks (250 mL capacity)
- Pear shaped flasks (40 mL, 50 mL capacity)
- Volumetric pipettes (3 mL capacity)
- Volumetric flasks (10 mL, 25 mL, 50 mL, 100 mL capacity)
- Autosampler and total recovery vials
- Amber bottles with Teflon® lined caps

AB Sciex API 5500 Series Triple Quad Mass Spectrometer with Agilent 1260 Series LC (LC-MS/MS), Analyst Data System Software

3.4 Test Systems

3.4.1 Source of the Test Systems

Natural surface water was collected from Whaley Pond, Lenoir, Co, NC. The test system was collected on November 10, 2015 and upon arrival at EAG Laboratories-Hercules, the inventory no. 2706W-042 was given. The water sample was stored refrigerated (typically < 4 °C) in the dark when not in use.

A sandy loam soil was collected at Hickman, CA and received on August 7, 2015. Upon arrival at EAG Laboratories-Hercules, the test system was assigned the inventory no. 2705W-033 and stored refrigerated (typically < 4 °C) in the dark when not in use.

3.4.2 Characterization of the Test Systems

Pond water and soil used in the study were characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). Total organic carbon was characterized at EAG Laboratories- Hercules. The characterization reports are presented in Appendix C.

3.5 Test Methods

The analytical method for the analysis of Fluthiacet-methyl (CGA-248757) and seven metabolites (CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059) in water by LC-MS/MS was independently validated at EAG Laboratories- Hercules based on the analytical method of a Syngenta report (Reference 1), provided by the Sponsor with some modifications.

Water samples were buffered to pH 5 with ammonium acetate and acetic acid and extracted using a Nexus solid phase extraction (SPE) cartridge with acetonitrile (ACN). Extracts were concentrated to near dryness via rotary evaporation over a water bath at 28 °C. Final extracts were redissolved in 10:90 (v:v) ACN/ 0.05 M ammonium acetate in water (pH adjusted to 5 with acetic acid) and analyzed by LC-MS/MS.

The analytical method for the analysis of Fluthiacet-methyl (CGA-248757) and seven metabolites (CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059) in soil by LC-MS/MS was independently validated at

EAG Laboratories- Hercules based on the analytical method of a Syngenta report (Reference 2), provided by the Sponsor with some modifications.

For the first soil independent laboratory validation (ILV) attempt, soil samples were extracted with 50:50 (v:v) ACN/ 0.05 M ammonium acetate in water, centrifuged and decanted in graduated cylinders containing 2 drops of ammonium hydroxide. Basic soil extracts were then cleaned up using SAX solid phase extraction (SPE) and eluate was collected in graduated cylinders containing acetic acid. Half of the acidic eluate was measured and concentrated via rotary evaporation to remove the ACN and 0.05 M ammonium acetate (pH = 5) in water was added to the concentrated samples followed by a second SPE clean up using Bond Elut Nexus cartridges and 9 mL ACN for the elution step. An aliquot (0.25 mL) of 0.05 M ammonium acetate (pH adjusted to 5 with acetic acid) was added to the eluate as a water trap for the analytes and ACN was removed (~ 0.5 mL remained) via rotary evaporation. Final concentrated extracts were diluted with 0.05 M ammonium acetate in water (pH = 5) so that final concentration was 10:90 (v:v) ACN/ 0.05 M ammonium acetate and analyzed by LC-MS/MS.

For the second soil ILV attempt, the same sample procedure was followed as mentioned above except that the elution volume of the Bond Elut Nexus SPE was modified from 9 mL to 7 mL. In addition, the HPLC analytical column was rejuvenated and the MS source was cleaned.

The percent recovery of analytes in water and soil was determined using external standardization where separate linear curves of calibration standards for each analyte and each monitored ion were analyzed along with the samples.

3.6 Preparation of Reference Substance Solutions

3.6.1 Preparation of Stock Solutions

Separate stock solutions of Fluthiacet-methyl (CGA-248757), CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 were prepared by weighing aliquots (approximately 10 mg) of the reference substances in weighing boats, transferring into 50 mL volumetric flasks, and diluting to the mark with ACN. Stock solutions were sonicated to ensure all solids have completely dissolved. Additional ACN was added as necessary to achieve a nominal concentration of 200 µg/mL after adjusting for the purity of the reference substances as follows:

Analyte	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical conc. (µg/mL) [1]	solvent	std ID
Fluthiacet-methyl (CGA-248757)	10.60	52.629	99.3	200	ACN	Stock
CGA-300402	10.32	51.187	99.2	200	ACN	Stock
CGA-300403	10.20	50.133	98.3	200	ACN	Stock
CGA-300404	10.99	51.488	93.7	200	ACN	Stock
CGA-327066	10.68	50.944	95.4	200	ACN	Stock
CGA-327067	10.94	50.488	92.3	200	ACN	Stock
CGA-330057	10.82	53.884	99.6	200	ACN	Stock
CGA-330059	10.56	50.846	96.3	200	ACN	Stock

[1]Theoretical conc. (µg/mL) = [weight (mg) x 1,000 µg/mg ÷ final volume (mL)] x [purity (%) ÷ 100]

The stock solutions were transferred into amber bottles and stored frozen (usually < -4 °C) when not in use. Additional stock solutions were prepared during the study as needed.

3.6.2 Preparation of Mixed Intermediate solutions in 50:50 (v:v) ACN/0.05 M ammonium acetate pH 5

A mixed intermediate solution at 10 µg/mL was prepared by volumetrically combining aliquots (5 mL) of each stock (200 µg/mL) into a 100 mL volumetric flask. Final solution was diluted to the mark with 50:50 (v:v) ACN/0.05 M ammonium acetate (pH = 5), vortexed to mix and transferred to an amber bottle. The mixed intermediate solution was stored either frozen (usually < -4 °C) or refrigerated (usually between 4 °C and 10 °C) when not in use. Additional mixed intermediate solutions were prepared during the study as needed.

3.6.3 Preparation of Mixed Intermediate solutions in 10:90 (v:v) ACN/0.05 M ammonium acetate pH 5

A mixed intermediate solution at 1.0 µg/mL was prepared by volumetrically measuring an aliquot (1 mL) of the mixed intermediate solution (10 µg/mL) described above into a 10 mL volumetric flask. Final solution was diluted to the mark with 10:90 (v:v) ACN/0.05 M ammonium acetate (pH = 5), vortexed to mix and transferred to an amber bottle.

A mixed intermediate solution at 0.1 µg/mL was prepared by volumetrically measuring an aliquot (1 mL) of the mixed intermediate solution (10 µg/mL) into a 100 mL volumetric flask. Final solution was diluted to the mark with 10:90 (v:v) ACN/0.05 M ammonium acetate (pH = 5), vortexed to mix and transferred to an amber bottle.

A mixed intermediate solution at 10 ng/mL was prepared by combining an aliquot (0.1 mL) of the mixed intermediate solution (0.1 µg/mL) described with 0.9 mL of 10:90 (v:v) ACN/0.05 M ammonium acetate (pH = 5) in a vial.

The mixed intermediate solutions were stored either frozen (usually < -4 °C) or refrigerated (usually between 4 °C and 10 °C) when not in use. Additional mixed intermediate solutions were prepared during the study as needed.

3.6.4 Preparation of Fortification Solutions

For the water validation set, a fortification solution containing a mixture of Fluthiacet-methyl and metabolites was prepared at 0.003 µg/mL by measuring an aliquot (0.075 mL) of the 1.0 µg/mL mixed intermediate solution and transferring into a 25 mL volumetric flask containing some 10:90 (v:v) ACN/0.05 M ammonium acetate (pH = 5). Final solution was diluted to the mark with same solvent. The fortification solution was transferred into an amber bottle and stored frozen (usually < -4 °C) when not in use.

For the soil validation set, a fortification solution containing a mixture of Fluthiacet-methyl and metabolites was prepared at 0.001 µg/mL by measuring an aliquot (0.1 mL) of the 1.0 µg/mL mixed intermediate solution and transferring into a 100 mL volumetric flask containing some 10:90 (v:v) ACN/0.05 M ammonium acetate (pH = 5). Final solution was diluted to the mark with same solvent. The fortification solution was transferred into an amber bottles and stored frozen (usually < -4 °C) or refrigerated (usually between 4 °C and 10 °C) when not in use. Additional fortification solutions were prepared during the study as needed.

3.6.5 Preparation of Solvent-Based Calibration Standard Solutions for Water Analysis

Six calibrants containing Fluthiacet-methyl and metabolites were prepared in 10: 90 (v:v) ACN: ammonium acetate (pH = 5) as follows: an appropriate volume of the 0.1 µg/mL mixed intermediate solution was transferred into separate 10 mL volumetric flasks containing some 10: 90 (v:v) ACN/ ammonium acetate (pH = 5). Final solutions were diluted to the mark with same solvent. The concentrations of Fluthiacet-methyl and metabolites ranged from 0.5 ng/mL to 10 ng/mL as shown below:

Solution used	Aliquot (mL)	Final volume (mL)	Theoretical conc. [1] (ng/mL) of Analyte
0.1 µg/mL mixed intermediate	0.050	10	0.5
0.1 µg/mL mixed intermediate	0.100	10	1.0
0.1 µg/mL mixed intermediate	0.250	10	2.5
0.1 µg/mL mixed intermediate	0.500	10	5.0
0.1 µg/mL mixed intermediate	1.000	10	10.0

[1]Theoretical conc. (ng/mL) = {[theoretical conc. soln used (µg/mL) x 1000 ng/µg x aliquot (mL)] ÷ final volume (mL)}

Note: solvent-based calibration solutions were used for the stability of the water extracts only.

Solvent-based calibration standard solutions were stored frozen (typically <- 4 °C) when not in use.

3.6.6 Preparation of Matrix-Based Calibration Standard Solutions for Water Analysis

Six calibration standard solutions containing Fluthiacet-methyl and metabolites were prepared in water matrix as follows: appropriate volumes of the 0.1 µg/mL mixed intermediate solution or 0.003 µg/mL Fortification solution were combined with aliquots of control (untreated) water sample extracts using glass precision syringes and mixed. The concentrations of Fluthiacet-methyl and metabolites ranged from 0.3 ng/mL to 10 ng/mL as shown below:

Solution used	Solution Aliquot (mL)	Control aliquot (mL)	Final volume (mL)	Theoretical conc (ng/mL)[1] of Analyte
0.003 µg/mL Fort Solution	0.025	0.225	0.25	0.3
0.003 µg/mL Fort Solution	0.040	0.210	0.25	0.5
0.003 µg/mL Fort Solution	0.080	0.170	0.25	1.0
0.1 µg/mL mixed intermediate	0.010	0.390	0.4	2.5
0.1 µg/mL mixed intermediate	0.020	0.380	0.4	5.0
0.1 µg/mL mixed intermediate	0.040	0.360	0.4	10.0

[1]Theoretical conc. (ng/mL) = {[theoretical conc. soln used (µg/mL) x 1000 ng/µg x soln aliquot (mL)] ÷ final volume (mL)}

3.6.7 Preparation of Matrix-Based Calibration Standard Solutions for Soil Analysis

Six calibration standard solutions containing Fluthiacet-methyl and metabolites were prepared in soil matrix as follows: appropriate volumes of the 10 ng/mL mixed intermediate solution or 0.001 µg/mL Fortification solution were combined with aliquots of control (untreated) soil sample extracts using glass precision syringes and mixed. The concentrations of Fluthiacet-methyl and metabolites ranged from 0.06 ng/mL to 0.8 ng/mL as shown below:

Solution used	Solution Aliquot (mL)	Control aliquot (mL)	Final volume (mL)	Theoretical conc (ng/mL)[1] of Analyte
0.001 µg/mL Fort Solution	0.015	0.235	0.250	0.06
0.001 µg/mL Fort Solution	0.020	0.230	0.250	0.08
0.001 µg/mL Fort Solution	0.040	0.210	0.250	0.16
0.001 µg/mL Fort Solution	0.050	0.200	0.250	0.2
10 ng/mL mixed intermediate	0.010	0.190	0.200	0.5
10 ng/mL mixed intermediate	0.020	0.230	0.250	0.8

[1]Theoretical conc. (ng/mL) = {[theoretical conc. soln used (µg/mL) x 1000 ng/µg x soln aliquot (mL)] ÷ final volume (mL)}

Note: additional matrix-based calibration standard solutions were prepared with different volumes but same concentration range for CGA-327067 reanalysis.

3.7 Preparation of Spiked Solutions for Matrix Effects Assessment

3.7.1 Preparation of Mixed Matrix-Based and Solvent-Based Standard Solutions for Water Analysis

A 10 ng/mL solvent-based standard solution containing Fluthiacet-methyl and metabolites was prepared by measuring 1 mL of the 0.1 µg/mL mixed intermediate and transferring into a 10 mL volumetric flask. Solution was diluted to the mark with 10: 90 (v:v) ACN: ammonium acetate (pH = 5).

A 1 ng/mL matrix-based standard solution containing Fluthiacet-methyl and metabolites was prepared by combining 0.9 mL of a water control extract and 0.1 mL of the 10 ng/mL solvent-based standard solution described above into an autosampler vial and mixed. Similar procedure was conducted for a solvent-based standard solution except that 0.9 mL of 10: 90 (v:v) ACN/ ammonium acetate (pH = 5) was used instead of the water control extract.

3.7.2 Preparation of Mixed Matrix-Based and Solvent-Based Standard Solutions for Soil Analysis

A 0.2 ng/mL matrix-based standard solution containing Fluthiacet-methyl and metabolites was prepared by combining 0.16 mL of a soil control extract and 0.04 mL of the 0.001 µg/mL Fortification solution into a high recovery vial and mixed. Similar procedure was conducted for a solvent-based standard solution except that 0.16 mL of 10: 90 (v:v) ACN/ ammonium acetate (pH = 5) was used instead of the soil control extract.

3.8 Stability of Standard Solutions

Stability of Fluthiacet-methyl and CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 was assessed by comparing a freshly prepared calibration standard solution (1 ng/mL) to a calibration standard solution of the same concentration that have been stored refrigerated (usually between 4 °C and 10 °C) after 46 days (43 days for Fluthiacel-methyl) and after 108 days.

3.9 Stability of Sample Extract Solutions

3.9.1 Stability of Water Extract Solutions

The stability of Fluthiacet-methyl and metabolites in the water extracts was assessed by reanalyzing one sample from each spike level (LOQ and 10X LOQ) after being stored refrigerated (usually between 4 °C and 10 °C) for 16 days. Quantitation of analytes in the water extracts was conducted by direct comparison (peak area response) to a freshly spiked water control extract at 1 ng/mL; only the quantitation ion transition for each analyte was used. Since the response of the water extracts had to be similar to that of the freshly spiked solution, 10X LOQ water sample was diluted 10-fold in water control (untreated sample) so that both extracts and spiked solution yielded a nominal assay concentration of 1 ng/mL.

Spiked water control extract was prepared by combining 0.08 mL of fortification solution (0.003 µg/mL) with 0.17 mL of water control extract (untreated sample) in a high recovery vial and mixed.

Two aliquots of water control extracts were spiked with CGA-327066 and CGA-327067 standards (0.003 µg/mL Fortification solution and 10 ng/mL solution) at LOQ and at 10X LOQ and analyzed against solvent-based calibration standard solutions (0.3 ng/mL – 10 ng/mL) as Time 0. Spiked water extracts were reanalyzed against the same standard solutions after 7 days of storage under refrigerated conditions.

3.9.2 Stability of Soil Extract Solutions

The stability of Fluthiacet-methyl and degradates in the soil extracts was assessed by reanalyzing one sample from each spike level (LOQ and 10X LOQ) after being stored refrigerated (usually between 4 °C and 10 °C) for 7 days. Quantitation of analytes in the soil extracts was conducted by direct comparison (peak area response) to freshly spiked soil control extracts equivalent to LOQ and 10X LOQ corresponding to 0.2 ng/mL and 2 ng/mL, respectively; only the quantitation ion transition for each analyte was used.

A spiked soil control extract at 0.2 ng/mL was prepared by combining 0.12 mL of fortification solution (0.001 µg/mL) with 0.48 mL of soil control extract (untreated sample) in a high recovery vial and mixed. A spiked soil control extract at 2 ng/mL was prepared by combining 0.12 mL of the 10 ng/mL mixed intermediate solution with 0.48 mL of soil control extract (untreated sample) in a high recovery vial and mixed.

3.10 Fortification Procedure

Fortification of untreated water and untreated soil samples was conducted at two fortification levels as shown below:

Test system (Matrix)	Fortification Level (µg/L) or (µg/kg)	Fortification volume (mL)	Solution used
Pond water (300 mL)	0.01 µg/L	1.0	0.003 µg/mL Fortification solution
	0.1 µg/L	0.3	0.1 µg/mL mixed intermediate solution
Soil (20 g)	0.05 µg/kg	1.0	0.001 µg/mL Fortification solution
	0.5 µg/kg	0.1	0.1 µg/mL mixed intermediate solution

Fortification was conducted to determine the percent recovery within the Independent Laboratory Validation. This procedure was performed in quintuplicate during Independent Laboratory Validation at each fortification level for each matrix.

3.11 Extraction Procedure for Fluthiacet-methyl and metabolites in Pond Water

1. Measure a 300-mL aliquot of sieved pond water into a 500-mL amber glass bottle. The water is buffered by adding 1 mL of 43.5% ammonium acetate/water, and 80 µL of acetic acid. Place cap on bottle and mix. Verify pH=5 using a pH strip.
2. Fortify samples as needed. Place cap on bottle and mix.
3. Precondition an Agilent Bond Elut Nexus SPE column (0.5 g / 12 mL) with 6 mL of methanol, followed by 10 mL of 0.05M ammonium acetate (pH = 5). Do not allow the SPE column to dry out between rinses.
4. Stop the flow, and add 2 mL of 0.05M ammonium acetate (pH = 5) into the cartridge.
5. Attach a 75-mL plastic reservoir on top of the SPE column.

6. Load the sample into the reservoir and let the sample pass through the SPE column by gravity, and discard the eluate.
7. Rinse the bottle twice with 10 mL of 0.05M ammonium acetate (pH=5), rinsing the sides of the reservoir as well. Allow the SPE to drain.
8. Elute the analytes from the SPE column with 10 mL of acetonitrile. Collect the eluate in a 40-mL pear shaped glass flask that has been calibrated to 3.0 mL. Add 0.5 mL of the 0.05M ammonium acetate (pH = 5) to the tube to act as a trap.
9. Calibrate by adding 3.0 mL of 0.05M ammonium acetate (pH = 5) using a volumetric pipette, and marking the meniscus with a fine-tipped permanent marker.
10. Remove acetonitrile until approximately 0.5 mL remains using a rotary evaporator (set the water bath to 28 °C, stair-step pressure from 125 to 35 mbar). Do not allow to go to dryness.
11. Remove the pear shaped glass flask from the rotary evaporator. Add 0.3 mL of acetonitrile and vortex for 1 minute. Dilute to the 3-mL mark with 0.05M ammonium acetate (pH = 5). Vortex for 30 seconds, then sonicate for approximately 1 minute. Transfer extract to a 4-mL glass vial for storage.
12. Aliquot solutions to autosampler vials for analysis; dilute as needed, using water control extracts. Analyze by LC-MS/MS.

3.12 Extraction Procedure for Fluthiacet-methyl and Metabolites in Soil

1. Weigh and record 20 ± 0.1 g of soil sample and place in a 250 mL disposable plastic bottle.
2. Fortify samples as needed.
3. Add 100 mL of 50:50 (v:v) acetonitrile:0.05M ammonium acetate. Place cap on bottle and shake vigorously for approximately 15 seconds. Place the bottle on a wrist-action shaker ad shake the sample for approximately one hour.
4. Remove the sample from the shaker and centrifuge at 8,000 rpm for 10 minutes (using the RC2-B centrifuge system).
5. Decant the solvent into a 100 mL graduated cylinder containing 2 drops of ammonium hydroxide.
6. Precondition an Agilent Bond Elut SAX SPE cartridge (1 g / 6 mL) with 10 mL of ammonium hydroxide-treated extraction solvent (add 25 µL of ammonium hydroxide for every 50 mL of extraction solvent used).

Note: Do not allow the SPE column to dry out between rinses.

7. Attach a 75-mL plastic reservoir on top of the SAX column.
8. Load the sample into the reservoir and let the sample pass through the SPE column and collect the eluate in a 250-mL graduated cylinder containing 50 µL of acetic acid.
9. Rinse the 100-mL graduated cylinder (Step 5) twice with 10 mL of ammonium hydroxide-treated extraction solvent. Allow the SPE to drain.
10. Homogenize the eluate. Quantitatively transfer half of the eluate into a 250-mL round bottom flask (portion A), and proceed with the extraction. Store the remaining eluate in an amber glass bottle (portion B).
11. Remove the acetonitrile from the sample via a rotary evaporator (set the water bath to 28 °C, stair step pressure from 150 to 50 mbar).
12. Dilute the sample with 10 mL of 0.05M ammonium acetate (pH = 5).
13. Precondition an Agilent Bond Elut Nexus SPE cartridge (500 mg / 12 mL) with 6 mL of methanol, followed by 10 mL of 0.05M ammonium acetate (pH = 5).

Note: Do not allow the SPE column to dry out between rinses.

14. Stop the flow, and add 2 mL of 0.05M ammonium acetate (pH = 5) into the cartridge.
15. Attach a 75-mL plastic reservoir on top of the Nexus column.
16. Load the sample from Step 12 into the SPE column by gravity, and discard the eluate.
17. Rinse the sample flask twice with 5 mL of 0.05M ammonium acetate (pH = 5), and transfer rinse to the SPE column.
18. Elute the analytes from the SPE column with 7 mL of ACN. Collect the eluate in a 50-mL pear shaped glass flask that has been calibrated to 2.5 mL. Add 0.25mL of the 0.05M ammonium acetate (pH = 5) to the tube as a trap.

Calibrate by adding 2.5 mL of 0.05M ammonium acetate (pH = 5) using a pipetman, and marking the meniscus with a fine-tipped permanent marker.

19. Remove acetonitrile until approximately 0.5 mL remains using a rotary evaporator (set the water bath to 28°C, stair-step pressure from 75 to 30 mbar).

Note: Do not allow to go to dryness.

20. Remove the pear shaped glass flask from the rotary evaporator. Add 0.25 mL of acetonitrile and vortex for 1 minute. Dilute to the 2.5-mL mark with 0.05M ammonium acetate (pH = 5). Vortex tube for 30 seconds, then sonicate for approximately 1 minute.

21. Aliquot solutions to autosampler vials for analysis. Dilute as needed, using soil control extracts. Analyze by LC-MS/MS.

3.13 Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analytical Method

3.13.1 LC conditions

Column: Phenomenex Develosil 3 µm, 140A, 150 mm x 3.0 mm
Guard column: Phenomenex C18, 4 mm x 2 mm (AJO-4286)
Column Temperature: 30°C
Injection Volume: 50 µL
Mobile Phase System: A) 0.1 % acetic acid in purified water
B) 0.1% acetic acid in ACN
Gradient:

Time (min.)	Flow Rate (µL/min.)	% A	% B
0.0	600	90	10
20.0	600	30	70
23.0	600	0	100
25.0	600	0	100
25.1	600	90	10
32.0	600	90	10

Approximate retention times:

- CGA-327067: 8.2 min
- CGA-330059: 10.4 min
- CGA-327066: 11.9 min
- CGA-300404: 13.1 min
- CGA-300403: 14.1 min
- CGA-330057: 16.4 min
- CGA-300402: 17.5 min
- CGA-248757: 20.7 min

3.13.2 MS conditions

Electrospray Ionization (ESI) in positive polarity mode
Acquisition scan mode: Scheduled Multiple Reaction Monitoring (MRM)

Period 1 settings: Experiment 1:

For quantitation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	CE (V)	CXP (V)
CGA-248757	404	404	10	15
CGA-300402	390	390	10	20
CGA-330057	420	347	28	16.3
CGA-300403	390	344	25.7	30.9
CGA-300404	406	347	27	14
CGA-327066	374	356	18.9	16.7
CGA-330059	406	346	21.8	14.9
CGA-327067	390	330	22	14.3

For confirmation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	CE (V)	CXP (V)
CGA-248757	404	344.1	30	16.6
CGA-300402	390	260	36	17.2
CGA-330057	420	217	54.2	16.9
CGA-300403	390	390	10	20
CGA-300404	406	362	19.8	16.1
CGA-327066	374	328	24	14.7
CGA-330059	406	344	20	14.5
CGA-327067	390	314	20	13.8

Representative Mass Spectrometer Settings:

	Period 1
CAD:	6.0
CUR:	25.0
GS1:	40.0
GS2:	40.0
IS:	2500.0
TEM:	500.0
DP:	50.0
EP:	10.0

3.14 LC-MS/MS Analyses

For the water validation sample set, water samples were analyzed interspersed between the mixed matrix-based calibration standard solutions in single injection. 10:90 (v:v) ACN/ ammonium acetate (pH = 5) was analyzed as the solvent blank. Water samples were analyzed in single injection. Matrix-based calibration standard solutions were analyzed in single injections except for at least one standard solution was reanalyzed at the end of the sequence as quality control standard (QC) during analysis to ensure good chromatography and consistent instrument performance.

For water extract stability analysis, a freshly spiked water control extract (1 ng/mL) was analyzed in duplicate followed by duplicate injections of one water sample at LOQ and one water sample at 10XLOQ after 16 days of storage (usually 4°C to 10°C). For water extract stability analysis for CGA-327066 and CGA-327067, solvent-based calibration standard solutions and stability samples (Time 0 and Time 7 days) were analyzed in single injection.

For the soil validation sample set, matrix-based calibration standard solutions were analyzed upfront from lowest concentration to highest concentration prior to the analysis of the soil samples in single injection. One standard solution was reanalyzed at least at the end of the sequence as QC. 10:90 (v:v) ACN/ ammonium acetate (pH = 5) was analyzed as the solvent blank. Soil samples were analyzed in single injection.

For soil extract stability analysis, a freshly spiked soil control extract (0.2 ng/mL) was analyzed in triplicate followed by triplicate injections of one soil sample at LOQ after 7 days of storage (usually 4 °C to 10 °C). Another freshly spiked soil control extract (2 ng/mL) was analyzed in triplicate followed by triplicate injections of one soil sample at 10X LOQ after 7 days of storage (usually 4 °C to 10 °C).

For stability of standard solutions (1 ng/mL), fresh and stored standard solutions (46 days (43 days for Fluthiacel-methyl) and 108 days) were analyzed at least once.

To assess if the responses of the calibration standard solutions had been affected by matrix samples either by signal suppression or enhancement, the mixed matrix-based and solvent-based standard solutions (1 ng/mL) for the water sample set were injected at least once; the mixed matrix-based and solvent-based standard solutions (0.2 ng/mL) for the soil sample set were injected in triplicate.

The stability of the signal was monitored by comparing the response (compound peak area) of a quality control standard injection with that of a comparable standard from the linear curve of the corresponding analyte within the sequence.

3.15 Methods of Calculation

3.15.1 Quantitation

Fluthiacet-methyl (CGA-248757), CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 were quantitated by the external standard method using a six-point linear curve regression for each matrix.

Separation of the analytes was achieved by LC-MS/MS with scheduled MRM mode. The analytes were identified by the coincidence of their retention times with their respective reference substances and MS characteristics. The quantitation of Fluthiacet-methyl and metabolites was conducted by peak area response of each compound relative to the theoretical concentration of the calibration standard solutions. The content of each compound in water samples and soil samples was quantitated against separate 1/x weighted linear curves ($y = mx + b$) of Fluthiacet-methyl (CGA-248757), CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 calibration standards where:

y = peak area
x = ng/mL compound injected
m = slope
b = intercept

Weighting of the calibration curve of each compound was applied so as to provide better curve fit at the lower concentration levels of each compound. The calculation of weighted curve equations (linear regression) and concentration (ng/mL) present in samples and calibration standards was conducted using Analyst® software.

The percent recovery of each analyte from fortified samples was determined by dividing the found amount (ng) of each compound (corrected for mean control contribution, if necessary) by the relevant theoretical fortified amount (ng).

3.15.2 Recovery in water

Recovery (%) = [(ng recovered – ng mean control) ÷ ng fortified)] x 100%

Where:
ng recovered = calculated concentration (ng/mL) x final volume (mL) x dilution factor
ng fortified = sample volume (mL) x fortification level (µg/L) x [1000 ng/µg ÷ 1000 mL/L]
Calculated concentration (ng/mL) was determined by Analyst® software

3.15.3 Recovery in soil

Recovery (%) = [(ng recovered – ng mean control) ÷ ng fortified)] x 100%

Where:
ng recovered = calculated concentration (ng/mL) x final volume (mL) x dilution factor
ng fortified = sample weight (g) x fortification level (µg/kg) x [(1000 ng/µg) ÷ (1000 g/kg)]
Calculated concentration (ng/mL) was determined by Analyst® software

3.15.4 LOQ theoretical/expected concentration (ng/mL) in water samples

LOQ (ng/mL) = (ng fortified÷ final volume mL) ÷ dilution factor

Where:
ng fortified = sample volume (mL) x fortification level (µg/L)

Example:
Compound = Fluthiacet-methyl at LOQ
Fortification level (µg/L) = 0.01 (equivalent to 0.01 ng/mL)
Sample volume (mL) = 300
Final volume (mL) = 3
Dilution factor= 1

Fluthiacet-methyl fortified (ng) = 300 mL x 0.01 ng/mL = 3
LOQ (ng/mL) = (3 ng ÷ 3 mL) ÷ 1 = 1.0 ng/mL

3.15.5 LOQ theoretical/expected concentration (ng/mL) in soil samples

LOQ (ng/mL) = [ng fortified x sample aliquot for clean-up (mL)] ÷ [final volume (mL) x dilution factor x volume of extraction solvent (mL) x aliquot dilution factor]

Where:
ng fortified = [sample weight (g) x fortification level (µg/kg)] x [1000 ng/µg ÷ 1000 g/kg]
Sample aliquot for clean-up (mL) = amount of solvent recovered from soil extraction
Dilution factor = performed on final extract immediately prior to analysis
Aliquot dilution factor = only half of the volume of each soil extract was taken through the full extraction after the SAX cartridge elution

Example:
Compound = Fluthiacet-methyl at LOQ
Fortification level (µg/kg) = 0.05 (equivalent to 0.05 ng/g)
Sample weight (g) = 20.0
Sample aliquot for clean-up (mL) = 94
Final volume (mL) = 2.5
Dilution factor = 1
Volume of extraction solvent (mL) = 100
Aliquot dilution factor = 2

Fluthiacet-methyl fortified (ng) = 20 g x 0.05 ng/g = 1.0
LOQ (ng/mL) = (1.0 ng x 94 mL) ÷ (2.5 mL x 1 x 100 mL x 2) = 0.188 ng/mL ~ 0.2 ng/mL

3.16 Calibration Range

The calibration curves were generated by Analyst® software for each compound in each validation set. The calibration ranges for Fluthiacet-methyl and seven metabolites in water and soil were as follows:

	Analyte	Concentration Range (ng/mL)	
		low	high
Pond water	Fluthiacet-methyl (CGA-248757)	0.3	10
	CGA-300402	0.3	10
	CGA-300403	0.3	10
	CGA-300404	0.3	10
	CGA-327066	0.3	10
	CGA-327067	0.3	10
	CGA-330057	0.3	10
	CGA-330059	0.3	10
Soil	Fluthiacet-methyl (CGA-248757)	0.06	0.8
	CGA-300402	0.06	0.8
	CGA-300403	0.06	0.8
	CGA-300404	0.06	0.8
	CGA-327066	0.06	0.8
	CGA-327067	0.06	0.8
	CGA-330057	0.06	0.8
	CGA-330059	0.06	0.8

3.17 Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.01 µg/L for Fluthiacet-methyl (CGA-248757), CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 in pond water as validated in this study. The LOQ for Fluthiacet-methyl and metabolites in pond water represented 1 ng/mL of each analyte in the respective analyte calibration standard solution using the current methodology.

The limit of quantitation (LOQ) was set at 0.05 µg/kg for Fluthiacet-methyl (CGA-248757), CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059 in soil as validated in this study. The LOQ for Fluthiacet-methyl and metabolites in soil represented 0.2 ng/mL of each analyte in the respective analyte calibration standard solution using the current methodology.

3.18 Limit of Detection

The limit of detection (LOD) was defined as approximately 30% of the LOQ. The calibration solutions were analyzed to confirm the desired LOD.

The confirmed LOD in pond water was 0.003 µg/L for all analytes which represented 0.3 ng/mL in the calibration standard solution using the current methodology

The confirmed LOD in soil was 0.015 µg/kg for all analytes which represented 0.06 ng/mL in the calibration standard solution using the current methodology.

3.19 Time Required for Completion of a Sample Set

For each matrix all validation samples can be efficiently handled and completed in one set. Each validation set consisted of a reagent blank, two controls (untreated samples) and five fortified samples at each level (LOQ and 10X LOQ).

Time required for one set from initiation of extraction until the completion of instrumental analysis and data evaluation for water matrix is as follows:

- Preparation of standard solutions, glassware cleaning, reagent preparation takes approximately 6 hours
- Sample preparation (extraction and clean-up) takes approximately 9 hours
- LC-MS/MS analysis and data processing (two transition ions for each analyte) take approximately 4 hours

TOTAL = approximately 19 hours (3 calendar days) for one analyst to complete a sample set to satisfy the validation requirements for water matrix.

Time required for one set from initiation of extraction until the completion of instrumental analysis and data evaluation for soil matrix is as follows:

- Preparation of standard solutions, glassware cleaning, reagent preparation takes approximately 6 hours
- Sample preparation (extraction and clean-up) takes approximately 16 hours

- LC-MS/MS analysis and data processing (two transition ions for each analyte) take approximately 4 hours

TOTAL = approximately 26 hours (4 calendar days) for one analyst to complete a sample set to satisfy the validation requirements for soil matrix.

3.20 Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression were the only statistical methods employed in this study.

4.0 RESULTS AND DISCUSSION

Independent Laboratory Validation for Fluthiacet-methyl (CGA-248757) and seven metabolites (CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059) in water and soil test systems was conducted with one reagent blank, two untreated samples (controls) and five samples spiked at each of the two fortification levels: LOQ and 10X LOQ.

The limit of quantitation (LOQ) was set at 0.01 µg/L for all eight analytes in pond water as validated in this study. The LOQ for Fluthiacet-methyl and metabolites in pond water represented 1 ng/mL of each analyte in the respective analyte calibration standard solution using the current methodology.

The limit of quantitation (LOQ) was set at 0.05 µg/kg for all eight analytes in soil as validated in this study. The LOQ for Fluthiacet-methyl and metabolites in pond water represented 0.2 ng/mL of each analyte in the respective analyte calibration standard solution using the current methodology.

Appendix A. Protocol, Protocol Amendment, and Protocol Deviation.

STUDY PROTOCOL

1.0 Title: Independent Laboratory Validation of Analytical Methods for the Determination of Fluthiacet-methyl and Seven Metabolites in Soil and Water.

2.0 Study Number:

PTRL West Study No. 2812W
FMC Tracking No. 2015AMT-FLU2297

4.0 Proposed Study Schedule:

Study Initiation Date: January 2016
Experimental Start Date: January 2016
Experimental Termination Date: April 2016
Study Completion Date: May 2016

5.0 Objectives: To independently validate Environmental Chemistry Methods (ECM) for the determination of Fluthiacet-methyl and seven metabolites in water and in soil by LC/MS-MS. The methods are described in "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-3300059 in Water by High Performance Liquid Chromatography with Mass Spectrometric Detection", Narong Chamkasem, Syngenta Crop Protection, Inc., project number 1215-00, January 9, 2001 and in "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-3300059 in Soil by High Performance Liquid Chromatography with Mass Spectrometric Detection", Narong Chamkasem, Syngenta Crop Protection, Inc., project number 122-99, March 22, 2001.

This study will be conducted according to US EPA Guidelines, OCSPP 850.6100. The study will be conducted to fulfill the re-registration requirements, and in compliance with Good Laboratory Practices (GLP) as stated in FIFRA 40 CFR Part 160.

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

6.0 Testing Facility:

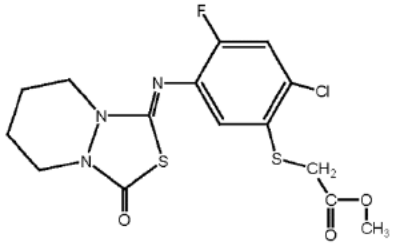
PTRL West (A division of EAG, Inc.)
625-B Alfred Nobel Dr.
Hercules, CA 94547

8.0 Test/Reference Substances

8.1 Fluthiacet-methyl (CGA-248757)

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]thio]-,methyl ester

CAS number: 117337-19-6
Molecular Formula: $C_{15}H_{15}ClFN_3O_3S_2$
Molecular Weight: 403.88 g/mole
Structure:



Lot number, purity, and expiration date will be provided in the final report

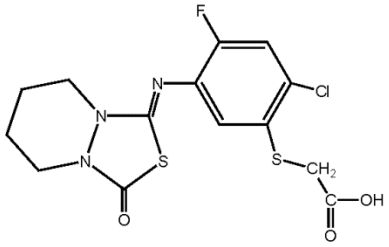
8.2 CGA-300402

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]thio]-

CAS number: 149253-65-6

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

Molecular Formula: $C_{14}H_{13}ClFN_3O_3S_2$
Molecular Weight: 389.85 g/mole
Structure:

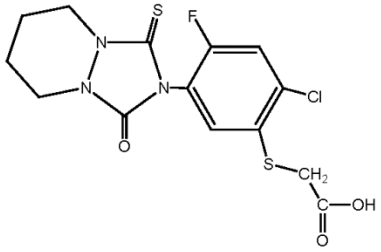


Lot number, purity, and expiration date will be provided in the final report

8.3 CGA-300403

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1-oxo-3-thioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2(3H)-yl)phenyl]thio]-

CAS number: not available
Molecular Formula: $C_{14}H_{13}ClFN_3O_3S_2$
Molecular Weight: 389.85 g/mole
Structure:



Lot number, purity, and expiration date will be provided in the final report

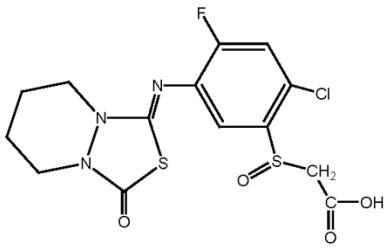
8.4 CGA-300404

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]sulfinyl]-

CAS number: not available
Molecular Formula: $C_{14}H_{13}ClFN_3O_4S_2$
Molecular Weight: 405.85 g/mole

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

Structure:

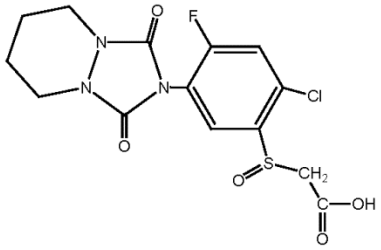


Lot number, purity, and expiration date will be provided in the final report

8.5 CGA-327067

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1,3-dioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2-(3H)-yl)phenyl]sulfinyl]-

CAS number: not available
Molecular Formula: $C_{14}H_{13}ClFN_3O_5S$
Molecular Weight: 389.79 g/mole
Structure:



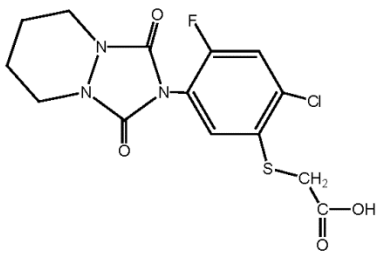
Lot number, purity, and expiration date will be provided in the final report

8.6 CGA-327066

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1,3-dioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2-(3H)-yl)phenyl]thio]-

CAS number: not available
Molecular Formula: $C_{14}H_{13}ClFN_3O_4S$
Molecular Weight: 373.79 g/mole
Structure:

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

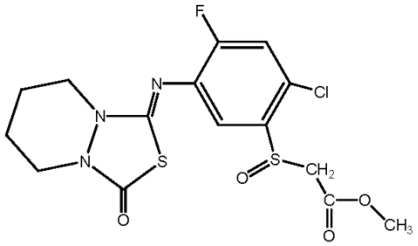


Lot number, purity, and expiration date will be provided in the final report

8.7 CGA-330057

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-3-oxo-1H-3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylidene)amino]phenyl]sulfinyl]-,methyl ester

CAS number: not available
Molecular Formula: $C_{15}H_{15}ClFN_3O_4S_2$
Molecular Weight: 419.88 g/mole
Structure:



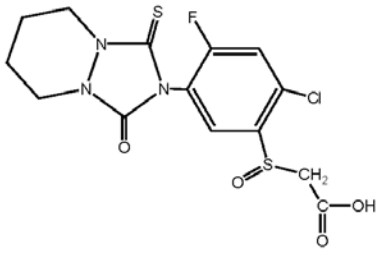
Lot number, purity, and expiration date will be provided in the final report

8.8 CGA-330059

Chemical Name: acetic acid, [[2-chloro-4-fluoro-5-[(tetrahydro-1-oxo-3-thioxo-1H-[1,2,4]triazolo[1,2-a]pyridazin-2(3H)-yl)phenyl]sulfinyl]-

CAS number: not available
Molecular Formula: $C_{14}H_{13}ClFN_3O_4S_2$
Molecular Weight: 405.85 g/mole
Structure:

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.



Lot number, purity, and expiration date will be provided in the final report

9.0 Characterization and Storage of Test, Control and Reference Substances

Assurance that the test, control and reference substances are properly characterized and stored is the responsibility of the test facility management. The sponsor will maintain information as to location of chemical synthesis (e.g., method of synthesis, spectral analysis, etc.) of the test, control and reference substances. These substances will be stored throughout the study period under the conditions specified by the Sponsor or supplier, to insure their stability. Stability information may be provided to PTRL West by the Sponsor to be included in the Final Report, as per 40 CFR Part 160.105. Storage conditions used will be documented in the study and/or facility records.

10.0 Safety: Precautions normally applied to the handling of pesticides. Safety Data Sheet (SDS) to be supplied by Sponsor or commercial source if available.

11.0 Receipt: Documentation in compliance with GLP regulations will be maintained for shipment, storage and use of test and reference standards.

12.0 Route of Administration: Known volumes of diluted test/reference substances of known concentration in solution will be spiked in the control matrices.

13.0 Justification of Route of Administration: To assess known quantity of residues introduced to the control matrices and recovered by the analytical method(s).

14.0 Test Systems (Matrices)

14.1 Water: The natural surface water is characterized by Agvise Laboratories, Inc., 604 Highway 15 West, Northwood, North Dakota. The water characterization (Agvise series 4) should include pH, calcium, magnesium, sodium, hardness, sodium adsorption ratio, dissolved organic carbon content and conductivity. The collection and characterization of the

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

test system to be used in this study is covered under another PTRL West study. Water characterization will be provided in the final report. The surface water will be stored refrigerated (usually 4°C to 10°C) when not in use.

14.2 Soil: The soil is characterized under another PTRL West study by Agvise Laboratories, Inc (604 Highway 15 West, Northwood, North Dakota). Soil should be characterized (at a minimum) using USDA classification with regard to texture (percent sand, percent silt, percent clay), pH and percent organic matter. Soil will be stored refrigerated (usually 4°C to 10°C) when not in use.

14.3 Justification: US EPA Guideline OCSPP 850.6100 requires the analytical methods for water and soil analyses to be validated independently.

14.4 Identification: untreated control water and soil samples will be labeled with PTRL Inventory numbers assigned by Compound Control. The Sample will be labeled with, at a minimum, the following information: PTRL study number, unique sample identification number and date/initials.

15.0 Study Design

15.1 Water Analytical Method: Fluthiacet-methyl and metabolites will be extracted and analyzed from nature surface water by a method provided by the Sponsor; "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-3300059 in Water by High Performance Liquid Chromatography with Mass Spectrometric Detection", Narong Chamkasem, Syngenta Crop Protection, Inc., project number 1215-00, January 9, 2001. The method is based on a solid phase extraction (SPE) of the water buffered to pH 5 with ammonium acetate and acetic acid, concentrating the organic partition by rotary evaporation, dissolving the residue in acetonitrile/0.05M ammonium acetate in water (pH adjusted to 5 with acetic acid), and analyzing final extract by liquid chromatography with tandem mass spectrometry LC/MS-MS.

15.2 Soil Analytical Method: Fluthiacet-methyl and metabolites will be extracted and analyzed from soil by a method provided by the Sponsor; "Analytical Method for the Determination of CGA-248757 and Its Degradates CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-3300059 in Soil by High Performance Liquid Chromatography with Mass Spectrometric Detection", Narong Chamkasem, Syngenta Crop Protection, Inc., project

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

number 122-99, March 22, 2001. The method is based on extraction of the soil with acetonitrile/0.05M ammonium acetate in water, SAX SPE, concentrating the eluate by rotary evaporation, SPE of the concentrated extract buffered to pH 5 with ammonium acetate, concentrating the eluate by rotary evaporation, dissolving the residue in acetonitrile/0.05M ammonium acetate in water (pH adjusted to 5 with acetic acid), and analyzing final extract by liquid chromatography with tandem mass spectrometry LC/MS-MS.

15.3 Linearity Curves: A minimum of five (5) data points in the concentration range that brackets the final samples.

15.4 Confirmation of Interferences: Signals in blank control samples should be <20% LOQ.

15.5 Recoveries: Method validation will be conducted with the analytes co-spiked as follows:

15.5.1 Water Sample Set:

1 regeant blank
2 untreated control samples
5 recoveries at the LOQ (0.01µg/L or 0.01 ppb)
5 recoveries at 10X LOQ (0.10 µg/L or 0.10 ppb)

15.5.2 Soil Sample Set: a complete set will be divided in two subsets for efficient handling. Each subset will include:

1 regeant blank
2 untreated control samples
5 recoveries at the LOQ (0.05µg/kg or 0.05 ppb) or
5 recoveries at 10X LOQ (0.50 µg/kg or 0.50 ppb)

Acceptable mean recoveries of each analyte for each spike level and each matrix will be between 70% and 120% with a relative standard deviation (RSD) ≤ 20%. Lower or higher recovery ranges may be deemed acceptable upon approval of the Sponsor.

The proposed LOQ in water will be 0.01 µg/L; the limit of detection (LOD) will be defined as 30% of the LOQ (0.003 µg/L).

The proposed LOQ in soil will be 0.05 µg/kg; the limit of detection (LOD) will be defined as 30% of the LOQ (0.015 µg/kg).

15.6 Quantitation: Quantitation will be conducted by peak area integration relative to an external calibration curve(s). The analytical methods will be based on LC/MS-MS. Quantitation will be assessed on one SRM (Selected Reaction Monitoring) transition ion per analyte in each test system.

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

15.7 Confirmation: Confirmation will be conducted by monitoring another SRM transition ion per analyte in each test system if possible.

15.8 Matrix effect: calibrants will be prepared in solvent and analyzed by LC/MS-MS (use quantitation ion transition only). At least one matrix based calibrant will also be prepared. The response of each calibrant type (matrix or solvent based) at the same concentration level will be compared to assess matrix effects. Solvent based calibrants will be used during the study if matrix effects are not significant (ie. signal enhancement < 120% recovery and signal suppression > 80% recovery). Matrix calibrants may be used if the signal enhancement or suppression is significant (> 20% deviation from solvent based calibrant response).

15.9 Stability of Standard Solutions: the stalibity of at least one calibration solution will be assessed (use quantitation ion transition only) after stored refrigerated (usually 4°C to 10°C) for ≥ 7 days against freshly prepared calibration solution.

15.10 Stability of Sample Extract Solutions: the stalibity of at least one sample extract solution from each spike level and each matrix will be assessed (use quantitation ion transition only) after stored refrigerated (usually 4°C to 10°C) for ≥ 7 days.

15.11 Proposed Statistical Methods: The following statistical methods will be applied to data generated for the validation study: linear or quadratic regression (may include 1/x weighting factor), standard deviation, relative standard deviation, confidence limits and averages.

15.12 Methods for Control of Bias: Methods to control bias will include analysis of untreated control matrices, interspersing calibration standards during analysis, and assaying multiple replicates at each fortification level.

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

17.0 Quality Assurance

At PTRL West, each project is assigned by its management to a member of the scientific staff with the appropriate education, training, and experience to accomplish the designed task. The QA Unit monitors the study to ensure that facilities, equipment, personnel, practices, procedures, records, and controls are designed to function in compliance with U.S. EPA FIFRA GLP Standards in 40 CFR Part 160, the protocol and applicable SOPs. The Quality Assurance Unit (QAU) will conduct inspections of this study, including at least one critical phase inspection, the study protocol, and a draft of the final report, and will provide the same written documentation of findings to management and the Study Director. Appropriate corrective action, if needed, will be instituted immediately. The QAU will issue a final report signed statement listing the date's inspections or audits were made and the date's findings were reported to management and the Study Director.

18.0 Records to be Maintained

All original data records and original final report will be the property of the Sponsor and will be temporarily archived at PTRL West and will be transferred to the Sponsor upon authorization. An exact copy of the Final Report and facility records (such as equipment use/calibration logs, etc.) will be retained in the archives at PTRL West for the period required by the EPA.

19.0 Final Report

A final report will be written for this study in accordance with PR 2011-3 format. The report will include, but is not limited to:

- The name and address of the Study Director and of the testing facility
- A statement of GLP compliance (any related documentation, such as chain of custody records, must be in the study records)
- A description/principle of the exact analytical conditions employed in the study
- Schematic diagram of the analytical methods
- Description of the instrumentation used and operating conditions
- Calibrant data and curves
- Example methods of calculation in a step-wise fashion
- All results from all validation sets analyzed
- Accuracy (mean and range of recoveries) and precision (relative standard deviation)
- Limit of detection (LOD) and limit of quantitation (LOQ)

Appendix A (continued). Protocol, Protocol Amendment, and Protocol Deviation.

- Mattrix effect results by comparison of solvent based calibrant(s) and matrix based calibrant(s)
- Stability results of standard and sample solutions
- Representative chromatograms from all ILV trials analyzed, including chromatograms of a standard and a control sample, and a chromatogram at each fortification level. Method (reagent) blanks should be included, if applicable. All analyte peaks should be clearly identified in all chromatograms.
- Time required for analysis of a sample set for each test system
- Description of any problems encountered
- A complete summary of any contact (communication) between the Study Director and the method developers or others familiar with the method, including the reasons for the contact, any changes in the method that resulted, and the time of this communication with respect to the progress of the study.
- Recommended changes to improve the method, where necessary

20.0 Protocol Deviations and Amendments

If modifications of this protocol are deemed necessary, a written statement of the change(s) and reason(s) proposed by the Sponsor or PTRL West, will be submitted to the other party. All agreed upon changes will be expressed in writing, signed and dated by the Study Director and the Sponsor. The signed changes will be amended to the protocol and included with the raw data. Deviations will be documented and submitted to the Study Director for acknowledgment by signature and reported to the Sponsor.

Appendix D. First ILV Attempt in Soil to Support Independent Laboratory Validation Study.

Extraction Procedure

The extraction procedure for Fluthiacet-methyl (CGA-248757) and seven metabolites (CGA-300402, CGA-300403, CGA-300404, CGA-327066, CGA-327067, CGA-330057, and CGA-330059) in soil for the first ILV attempt was the same as described in the materials and methods section of the report except that 9 mL ACN was used as elution volume instead of 7 mL. Due to the low recovery of CGA-327066 and CGA-327067 in the first ILV attempt, the extraction procedure was modified in the second attempt.

Stability of Soil Extract Solutions

The stability of Fluthiacet-methyl and degradates in the soil extracts was assessed by reanalyzing one sample from each spike level (LOQ and 10X LOQ) after being stored refrigerated (usually between 4°C and 10°C) for 8 days. Quantitation of analytes in the soil extracts was conducted by direct comparison (peak area response) to a freshly spiked soil control extract at 0.2 ng/mL; only the quantitation ion transition for each analyte was used. Since the response of the soil extracts had to be similar to that of the freshly spiked solution, 10XLOQ water sample was diluted 10-fold in soil control (untreated sample) so that both extracts and spiked solution yielded a nominal assay concentration of 0.2 ng/mL.

A spiked soil control extract at 0.2 ng/mL was prepared by combining 0.1 mL of fortification solution (0.001 µg/mL) with 0.4 mL of soil control (untreated sample) in a high recovery vial and mixed.

For the soil extracts of the first ILV attempt, results indicated to be stable for 8 days under refrigerated conditions (usually between 4°C and 10°C) except for CGA-327067.

Reagent Blank and Control Interferences

Negligible interferences (< LOD) or no residues were detected in the untreated samples (controls) of soil matrix and reagent blank.