Study Title

Independent Laboratory Validation of the Analytical Method for Determination of Fluometuron and its Metabolites (Des-methyl-Fluometuron and CGA 72903) in Soil by LC-MS/MS

Test Guidelines

OCSPP 850.6100
OCSPP 860.1340
SANCO/825/00 rev. 8.1 (2010)

1.0 INTRODUCTION

This independent laboratory validation (ILV) study is required by the U.S. EPA under the Guideline for Environmental Chemistry Method and Associated Independent Laboratory Validations OCSPP No. 850.6100 (U.S. EPA, 2012), Residue Analytical Methods OCSPP No. 860.1340 (U.S. EPA, 1996), as well as satisfies SANCO/825/00 rev. 8.1 (EC, 2010), to confirm that the original analytical method, developed by one laboratory, can be independently validated by a second laboratory. This analytical method was validated by fortification of soil with fluometuron and metabolites at the limit of quantification (LOQ, 0.0100 mg/kg) and 10X LOQ (0.100 mg/kg) concentration levels.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

The objective of this study is to confirm that the analytical method for fluometuron and its metabolites (des-methyl-fluometuron and CGA72903) in soil, developed by one group, can be independently validated by a second group in the absence of major interaction between the two. This study was performed following the Smithers Viscient protocol entitled "Independent Laboratory Validation of the Analytical Method For Determination of Fluometuron and its metabolites (Des-methyl-Fluometuron and CGA 72903) in Soil by LC MS/MS" (Appendix 1). The methods described in this protocol meet the requirements specified in the OCSPP Guideline 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA, 2012), OSCPP Guideline 860.1340: Residue Analytical Method (U.S. EPA, 1996), and SANCO/825/00 rev. 8.1 (EC, 2010).

2.2 Test Substances

The test substance, fluometuron, was received on 8 February 2018 from Chem Service, Inc., West Chester, Pennsylvania. The following information was provided:

Name:	Fluometuron
Lot No.:	6904200
CAS No.:	2164-17-2
Purity:	99.5% (Certificate of Analysis, Appendix 2)
Expiration Date:	31 July 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 9278) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, des methyl fluometuron, was received on 3 April 2018 from ADAMA Agan Ltd., Northern Industrial Zone, Ashdod, Israel. The following information was provided:

Name:	Des methyl fluometuron
Synonym:	Des-methyl-fluometuron
Lot No.:	FLMT(5)-BP1-918(V2)
CAS No.:	3032-40-4
Purity:	99.5% (Certificate of Analysis, Appendix 2)
Expiry Date:	30 September 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 9356) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

The test substance, 3-(Trifluoromethyl)aniline, was received on 9 February 2018 from Sigma-Aldrich Inc., Milwaukee, Wisconsin. The following information was provided:

Name:	3-(Trifluoromethyl)aniline
Synonym:	CGA 72903

Batch No.:	SHBH3630V
CAS No.:	98-16-8
Purity:	99.7% (Certificate of Analysis, Appendix 2)
Expiration Date:	Not listed

Upon receipt at Smithers Viscient, the test substance (SMV No. 9282) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

Determination of stability and characterization, verification of the test substance identities, maintenance of records on the test substances, and archival of samples of the test substances are the responsibility of the Study Sponsor.

2.3 Reagents

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

2.4 Equipment

1.	Instrument:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V source Shimadzu SIL-20ACHT autoinjector Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu CTO-20A column compartment Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balances:	Mettler Toledo XSE205DU; Mettler PG-2002-S
3.	Moisture balance:	Mettler Toledo Moisture Balance HB43-S
4.	Shaker tables:	VWR Shaker Table 3500; VWR Standard Analog Shaker 3500STD
5.	Centrifuge:	Thermo Scientific Sorvall Legend XFR Centrifuge

6. Laboratory equipment: Volumetric flasks, graduated cylinders, disposable glass pipets, positive displacement pipets, stir bars, stir plates, vortexer, 50-mL Nalgene centrifuge tubes, clear vials with blue snap caps, and amber glass bottles with Teflon-lined caps

2.5 Test Systems

The test systems evaluated during this study were soils representative of the type of matrix this method was intended to analyze. The soils used for this ILV analysis were sandy loam soil (SMV 15Feb17 Soil-A) and loamy sand soil (SMV 03Jan18 Soil-A). The soil characterization data are listed in the table below.

Soil Type	Percent Sand, Silt, Clay	Bulk Density (gm/cc)	Cation Exchange Capacity (meq/100 g)	Percent Organic Matter (Walkley Black)	Percent Moisture at 1/3 Bar	pH in 1:1 Soil:Water Ratio
Sandy Loam	73, 22, 5	1.20	9.30	0.48	18.2	7.8
Loamy Sand	42, 40, 18	1.11	10.5	1.7	22.9	7.3

NOTE: GLP soil characterization was conducted at Agvise Laboratories, Northwood, North Dakota.

2.6 Preparation of Liquid Reagent and Mobile Phase Solutions

The volumes listed in this section were those used during the independent laboratory validation. For future testing, the actual volumes used may be scaled up or down as necessary.

An 80/20 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 1200 mL of acetonitrile and 300 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 50/50 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 100 mL of acetonitrile and 100 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 0.25% formic acid in acetonitrile mobile phase solution was typically prepared by adding 5.00 mL of formic acid to 2000 mL of acetonitrile. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for 10 minutes.

A 0.25% formic acid in purified reagent water mobile phase solution was typically prepared by adding 5.00 mL of formic acid to 2000 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for 10 minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water. The solution was mixed well before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

Primary stock solutions were typically prepared as described in the table below.

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
9278B	0.02518	0.02505	Acetonitrile	25.0	1000	Fortification sub-stock solution and secondary stock solution
9356B	0.02514	0.02501	Acetonitrile	25.0	1000	Fortification sub-stock solution and secondary stock solution
9282B	0.02512	0.02504	Acetonitrile	25.0	1000	Fortification sub-stock solution and secondary stock solution

Secondary stock solutions were typically prepared as described in the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
9278B	1000	0.500	50.0	Acetonitrile	9278B-1	10.0	Sub-stock solution
9356B	1000	0.500	50.0	Acetonitrile	9356B-1	10.0	Sub-stock solution
9282B	1000	0.500	50.0	Acetonitrile	9282B-1	10.0	Sub-stock solution

Sub-stock solutions were typically prepared as described in the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
9278B	1000	0.100	10.0	50/50 acetonitrile/ purified reagent water (v/v)	Tech Mix-1	10.0	Fortification sub-stock solution and 10X LOQ recovery samples
9356B		0.100					
9282B		0.100					
Tech Mix-1	10.0	1.00	10.0	50/50 acetonitrile/ purified reagent water (v/v)	Tech Mix-2	1.00	LOQ-level recovery samples
9278B-1	10.0	1.00	10.0	50/50 acetonitrile/ purified reagent water (v/v)	Ana Mix-1	1.00	Sub-stock solution
9356B-1		1.00					
9282B-1		1.00					
Ana Mix-1	1.00	1.00	10.0	50/50 acetonitrile/ purified reagent water (v/v)	Ana Mix-2	0.100	Sub-stock solution and calibration standards
Ana Mix-2	0.100	1.00	10.0		Ana Mix-3	0.0100	Sub-stock solution and calibration standards
Ana Mix-3	0.0100	1.00	10.0		Ana Mix-4	0.00100	Calibration standards

All stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh daily and stored refrigerated for possible future use.

2.8 Preparation of Calibration Standards

2.8.1 Solvent-Based Calibration Standards

Standards were prepared in 50/50 acetonitrile/purified reagent water (v/v) using the 1.00, 10.0, and 100 µg/L sub-stock solution according to the table below. Following fortification, each solution was vortex-mixed for 15 seconds, then standards were transferred to clear vials with blue snap caps for analysis.

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Mix-4	1.00	1.00	10.0	0.100	Std 1
Ana Mix-3	10.0	0.200	10.0	0.200	Std 2
		0.500	10.0	0.500	Std 3
		1.00	10.0	1.00	Std 4
Ana Mix-2	100	0.200	10.0	2.00	Std 5
		0.500	10.0	5.00	Std 6
		1.00	10.0	10.0	Std 7

2.8.2 Matrix Effects Calibration Standards

In an effort to observe any potential matrix effects, an aliquot of control sample final dilution was fortified with the 100 µg/L sub-stock solution in triplicate and analyzed at each transition. These matrix-matched standards were compared to non-matrix-matched (solvent) standards fortified at the same concentration.

Sandy Loam Soil

Matrix-Matched Standards

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Mix-2	100	0.0250	5.00[a]	0.500	MM-Std A
		0.0250	5.00[a]	0.500	MM-Std B
		0.0250	5.00[a]	0.500	MM-Std C

[a] Diluted with final dilution of Control matrix blank 14090-6107-02

Non Matrix-Matched Standards

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Mix-2	100	0.0250	5.00[a]	0.500	SS-Std A
		0.0250	5.00[a]	0.500	SS-Std B
		0.0250	5.00[a]	0.500	SS-Std C

[a] Diluted with 50/50 acetonitrile/purified reagent water (v/v)

Loamy Sand Soil

Matrix-Matched Standards

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Mix-2	100	0.0250	5.00[a]	0.500	MM-Std D
		0.0250	5.00[a]	0.500	MM-Std E
		0.0250	5.00[a]	0.500	MM-Std F

[a] Diluted with final dilution of Control matrix blank 14090-6107-15

Non Matrix-Matched Standards

Fortifying Stock ID	Stock Concentration (µg/L)	Fortification Volume (mL)	Final Volume (mL)	Standard Concentration (µg/L)	Sample ID
Ana Mix-2	100	0.0250	5.00[a]	0.500	SS-Std D
		0.0250	5.00[a]	0.500	SS-Std E
		0.0250	5.00[a]	0.500	SS-Std F

[a] Diluted with 50/50 acetonitrile/purified reagent water (v/v)

2.9 Sample Fortification and Preparation

2.9.1 Sandy Loam Soil

Twelve aliquots of sandy loam soil (10.0 g dry weight, equivalent to 11.10 g wet weight based on measured percent moisture of 9.69%) were weighed into individual 250-mL screw cap glass bottles. Five replicates were dosed with the 1.00 mg/L mixed sub-stock solution and five aliquots were dosed with the 10.0 mg/L mixed sub-stock solution to obtain concentrations of 0.0100 and 0.100 mg/kg, respectively. Two aliquots were left unfortified to serve as controls and an additional sample was extracted using only solvents as a reagent blank. The dosing procedure is detailed in the following table.

Sample ID 14090-6107-	Sample Type	Stock ID	Fortifying Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Soil Weight (g)	Dry Weight (g)	Nominal Concentration (mg/kg)
1	Reagent Blk	NA[a]	NA	NA	NA	NA	0.00
2 & 3	Control	NA	NA	NA	11.10	10.0	0.00
4, 5, 6, 7, & 8	LOQ	Tech Mix-2	1.00	0.100	11.10	10.0	0.0100
9, 10, 11, 12, & 13	10X LOQ	Tech Mix-1	10.0	0.100	11.10	10.0	0.100

[a] NA = Not Applicable

2.9.2 Loamy Sand Soil

Twelve aliquots of loamy sand soil (10.0 g dry weight, equivalent to 10.93 g, wet weight based on measured percent moisture of 8.52%) were weighed into individual 250-mL screw cap glass bottles. Five replicates were dosed with the 1.00 mg/L mixed sub-stock solution and five aliquots were dosed with the 10.0 mg/L mixed sub-stock solution to obtain concentrations of 0.0100 and 0.100 mg/kg, respectively. Two aliquots were left unfortified to serve as controls and an additional sample was extracted using only solvents as a reagent blank. The dosing procedure is detailed in the following table.

Sample ID 14090-6107-	Sample Type	Stock ID	Fortifying Stock Concentration (mg/L)	Fortification Volume (mL)	Wet Soil Weight (g)	Dry Weight (g)	Nominal Concentration (mg/kg)
14	Reagent Blk	NA[a]	NA	NA	NA	NA	0.00
15 & 16	Control	NA	NA	NA	10.93	10.0	0.00
17, 18, 19, 20, & 21	LOQ	Tech Mix-2	1.00	0.100	10.93	10.0	0.0100
22, 23, 24, 25, & 26	10X LOQ	Tech Mix-1	10.0	0.100	10.93	10.0	0.100

[a] NA = Not Applicable

2.10 Extraction and Dilution of Fortified Recovery Samples

Samples were extracted once with 100 mL of 80/20 acetonitrile/purified reagent water (v/v). Samples were placed on a shaker table for approximately two hours at 300 rpm, (see Method Differences). A 25.0-mL aliquot of each fortified recovery sample was transferred into separate 50 mL Nalgene centrifuge tubes and centrifuged at 7800 rpm for five minutes. The

recovery sample extracts were further diluted into the calibration standard range with purified reagent water. The extraction and dilution procedures are detailed in the following table.

Sandy Loam Soil

Sample ID 14090-6107-	Sample Type	Nominal Concentration (mg/kg)	Dry Soil Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
1	Reagent Blk	0.00	NA[c]	100	100	5.00	10.0	20.0
2	Control	0.00	10.0	100	100	10.0[d]	20.0[d]	20.0
3	Control	0.00	10.0	100	100	5.00	10.0	20.0
4, 5, 6, 7, & 8	LOQ	0.0100	10.0	100	100	5.00	10.0	20.0
9, 10, 11, 12, & 13	10X LOQ	0.100	10.0	100	100	5.00	10.0	20.0

[a] Extraction Solvent: 80/20 acetonitrile/purified reagent water (v/v)
[b] Dilution solvent: purified reagent water
[c] NA = Not Applicable
[d] Volume increased to make enough control matrix blank for matrix-matched calibration standards.

Loamy Sand Soil

Sample ID 14090-6107-	Sample Type	Nominal Concentration (mg/kg)	Dry Soil Weight (g)	Extract Volume[a] (mL)	Final Volume[a] (mL)	Dilution (mL)	Final Volume[b] (mL)	Dilution Factor
14	Reagent Blk	0.00	NA[c]	100	100	5.00	10.0	20.0
15	Control	0.00	10.0	100	100	10.0[d]	20.0[d]	20.0
16	Control	0.00	10.0	100	100	5.00	10.0	20.0
17, 18, 19, 20, & 21	LOQ	0.0100	10.0	100	100	5.00	10.0	20.0
22, 23, 24, 25, & 26	10X LOQ	0.100	10.0	100	100	5.00	10.0	20.0

[a] Extraction Solvent: 80/20 acetonitrile/purified reagent water (v/v)
[b] Dilution solvent: purified reagent water
[c] NA = Not Applicable
[d] Volume increased to make enough control matrix blank for matrix-matched calibration standards.

2.11 LC-MS/MS Instrumental Conditions

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

LC Parameters:

Column:	Agilent Eclipse XDB-C18, 5 µm, 150 × 4.6 mm
Mobile Phase A:	0.25% Formic acid in water
Mobile Phase B:	0.25% Formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.50	0.500	50.0	50.0
5.00	0.500	5.00	95.0
7.00	0.500	5.00	95.0
7.01	0.500	50.0	50.0
9.00	0.500	50.0	50.0

Run Time: 9.0 minutes
Injector Wash Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 20.0 µL
Retention Times:

Analyte	Approximate Retention Time (min)
Fluometuron	5.4
Des-methyl-fluometuron	4.8
CGA 72903	5.7

MS Parameters:

Instrument: MDS Sciex API 5000 mass spectrometer
Ionization Mode: Positive (+) ESI
Ion Spray Voltage: 5000 V
Scan Type: MRM
Dwell Time: 100 msec
Source Temperature: 600 °C
Curtain Gas: 20.0
Ion Source – Gas 1 / Gas 2: 60.0 / 60.0
Collision Gas: 6.00
Collision Cell Entrance Potential: 10.0
Collision Cell Exit Potential: 15.0
Declustering Potential: 50.0
Resolution (Q1/Q3): Low/Low

Analyte	Transition	Q1/Q3 Mass (Da/Da)	Collision Energy
Fluometuron	Primary	233/160	38.0
	Confirmatory	233/72	35.0
Des-methyl-fluometuron	Primary	219/162	25.0
	Confirmatory	219/142	36.0
CGA 72903	Primary	162/142	28.0
	Confirmatory	162/93	33.0

2.11.1 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, every three to five injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.11.2 Method Differences

The analytical method used for fluometuron and its metabolites in this independent laboratory validation followed the procedures described in the original method validation. The analytical method used for fluometuron and its metabolites in this independent laboratory validation required the following minor modifications from the original method validation.

- The validated method did not specify shaking speed. In this study, acceptable results were obtained when samples were placed on an orbital shaker table at 300 rpm for two hours.
- Mass spectrometer parameters were optimized for sensitivity and linearity, as necessary.
- During the LC-MS/MS analysis, 0.25% formic acid in water and 0.25% formic acid in acetonitrile instead of 0.5% formic acid, was used. Typically, addition of a modifier in the mobile phases is done to get better ionization and chromatographic separation of the peaks. The current ILV results show that there is adequate sensitivity in the lowest standard and chromatographic pattern of fluometuron and its metabolites similar to the original validation. Additionally, accuracy, precision, and linearity met the acceptance criteria; therefore, this method difference did not have any impact on the results or interpretation of this study.

2.12 Evaluation of Accuracy, Precision, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the LOQ and 10X LOQ recovery samples. Recoveries of 70.0 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the LOQ and 10X LOQ recovery samples. The retention time should have an RSD of less than or equal to 2%. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. The specificity of the method was determined by examination of the control samples for peaks at the same retention time as fluometuron and metabolites which might interfere with the quantitation of the analytes. Interferences with peak areas that are less than 30% of the LOQ are not considered significant. The linearity of the method was determined by the correlation coefficient (r), y-intercept, and slope of the regression line. A 1/x weighted linear regression was used for the LC-MS/MS analysis. The calibration curves were evaluated based on the correlation coefficient and the recoveries of the calibration standards. The signal response data should have an intercept close to zero and a correlation coefficient (r) not less than 0.995. The precision of the method at the LOQ was reported in terms of the coefficient of variation of the observed recovery values.

2.13 Limit of Quantitation (LOQ)

The method was validated at the LOQ. This was defined as the lowest fortification level, with mean recoveries ranging between 70 and 120%, and a relative standard deviation not exceeding 20%. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.14 Limit of Detection (LOD)

The LOD was calculated using three times the signal-to-noise value of the control samples.

The LOD was defined as the lowest concentration in test samples which can be detected based on the concentration of the low calibration standard and the dilution factor of the control solutions.

2.15 Communications

Communications occurred with the Study Monitor to discuss items including: approval of the protocol and method, timing updates, and the results of the first attempt of the ILV.

A

2.16 Time Required for Analysis

There were two soil matrices investigated in this ILV. Each soil matrix investigation included one set of samples used for LC-MS/MS analysis. Each set of samples consisted of 10 fortified samples, two unfortified samples, one reagent blank, six matrix effects standards, and seven calibration standards (26 samples total). A single analyst completed a set of 27 samples in one working day (eight hours) with LC-MS/MS analysis performed overnight (approximately seven hours) per sample set.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and

intercept from the linear regression analysis with 1/x weighting, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \quad y = mx + b$$

$$(2) \quad DC(x) = \frac{(y - b)}{m}$$

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

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample weight)
A	=	analytical result (mg/kg), concentration in the original sample

NOTE: A 1/x weighting was used for calibration curves and sample quantitation using Analyst software, version 1.6.3.

The LOD is defined as the lowest concentration that can be detected by this method in test solution samples. The LOD is calculated (equation 4) based on the concentration of the low calibration standard and the dilution factor of the control samples.

$$(4) \quad LOD = LOD_{LCAL} \times DF_{CNTL}$$

where:

LOD_{LCAL}	=	lowest concentration calibration standard (0.100 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 20.0)
LOD	=	Limit of detection reported for the analysis (0.100 µg/L × 20.0 = 2.00 µg/L)

APPENDIX 1 – STUDY PROTOCOL

Study No.: 14090.6107

Independent Laboratory Validation of the Analytical Method for Determination of Fluometuron and its metabolites (Des-methyl-Fluometuron and CGA72903) in Soil by LC/MS/MS

1.0 INTRODUCTION

The purpose of this study is to confirm that an analytical method, developed by one group, can be independently validated by a second group in the absence of major interaction between the two. This study is required by EPA under guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174], and guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation, and must also satisfy SANCO/825/00 rev. 8.1: Guidance Document on pesticide residue analytical methods. Independent labs are allowed to analyze three sample sets in order to validate the method as written. A complete set of samples should consist of, at a minimum, a reagent blank, two un-spiked matrix control samples, five matrix control samples fortified at the limit of quantification (LOQ), and five matrix control samples fortified at 10X LOQ for each distinct matrix. A complete set may include more than thirteen samples depending on the number of reagents, un-fortified and fortified control matrix samples. It may be necessary, however, to divide a complete set into two subsets for efficient handling. Each subset should contain a reagent blank, two un-fortified matrix control samples, and five matrix control samples fortified at the LOQ or 10X LOQ.

If the performance data on the first set of samples at any of the required spiking levels is unsuccessful, the independent laboratory may contact the registrant to clarify the directions given in the method. Any contact with the registrant or developers during the method validation must be documented in writing in the final report submitted by the independent laboratory. If the independent laboratory cannot generate performance data that is similar to the registrant's or developers' after the second set of spiked samples, the independent laboratory may contact the registrant to further clarify the directions given in the method. If the independent laboratory cannot generate performance data that is similar to the registrant's or developers' after the third set, the method should be failed and a report will be sent to the registrant explaining why the method failed. The registrant should then decide whether to repeat the independent laboratory validation at another laboratory, further develop the method or withdraw it. A maximum of three sample sets are used by an independent laboratory to validate the method as written. A successful ILV trial will require adequate results on at least one complete set of samples on a given matrix.

The purpose of this protocol is to perform an ILV for the LC/MS/MS analytical method used to determine the test substance(s) in two soil types. The analytical method will be validated for fluometuron and its metabolites (des-methyl-fluometuron and CGA72903) with regards to accuracy, precision, signal response, selectivity, and limits of quantitation.

2.0 OBJECTIVE

The objective of this study is to confirm that the analytical method for fluometuron and its metabolites (des-methyl-fluometuron and CGA72903) in soil, developed by one group, can be independently validated by a second group in the absence of major interaction between the two.

3.0 JUSTIFICATION OF THE TEST SYSTEM

The method validation described in this protocol are designed to conform to EPA guideline OCSPP 860.1340: Residue Analytical Method [EPA 712-C-96-174], OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation, and SANCO/825/00 rev. 8.1: Guidance Document on pesticide residue analytical methods. The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40CFR160 and as accepted by the OECD principles on GLP.

4.0 MATERIALS

4.1 Test Substance

Upon arrival at Smithers Viscient, the test and reference substance(s) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

5.0 TEST SYSTEM IDENTIFICATION

Test solution preparation will be documented on data forms which include the amount of test substance, the volume or mass of the test solution, lot, batch or other sample designation of the test substance and date the solution was prepared. Individual sample containers will be labeled with the study number and a unique ID number.

6.0 ANALYTICAL METHOD

The analytical method to be used during the ILV is, "Validation of an Analytical Method for Determination of Fluometuron and its metabolites (Des-methyl-Fluometuron and CGA72903) in Soil", Sponsor Study No. 90014919, Study Code S12-00098, July 12, 2012".

7.0 VALIDATION DESIGN

The test design will consist of two soil types (identified in raw data and final report) fortified with the test substances at two concentrations with five replications for each fortification level. The control matrix for the validation will be untreated soils. The validation study levels (approximate concentrations) for test substances are:

Procedural blank-reagent blank	0.0 mg/kg
Matrix blank-control matrix	0.0 mg/kg
Control matrix fortified at LOQ	0.010 mg/kg
Control matrix fortified at 10 x LOQ	0.10 mg/kg

7.1 Accuracy and Precision

The accuracy of the analytical method will be determined by applying the method to five samples at the LOQ and five samples at 10X LOQ for each test substance. The accuracy will be reported in terms of percent recovery and the difference between the mean determined and the theoretical value. Overall mean recoveries of 70 to 120% of nominal are acceptable.

The precision will be calculated for the fortified samples in terms of the standard deviation (SD) and relative standard deviation (RSD or coefficient of variation (CV)) calculated for the retention time, peak area based quantitation (i.e., µg/L), and the observed recovery values. The retention time should have a RSD of less than or equal to 2%. The RSD of the peak area based quantitation (i.e., µg/L) should be less than or equal to 20%. The RSD of the recovery values should be less than or equal to 20% as well.

7.2 Specificity

The specificity of the method will be determined by applying the method to two un-fortified matrix control samples. Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte peak of interest. Peaks attributable to test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification. The limit of detection (LOD) will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples. Interferences with peak areas that are less than 30% at LOQ are not considered significant.

7.3 Regression Analysis

The linearity of the method will be determined by preparing a calibration curve with a minimum of five standards to encompass the test concentration ranges after sample processing. Other types of regression analyses (e.g. polynomial or logarithmic) may also be used if necessary. A smaller, larger, or shifted range may be necessary if achievable. The range will be documented in the study records and final report.

The calibration data will be subjected to a regression analysis; a plot of the analyte concentration versus the detector response will be included in the report along with the correlation coefficient, y-intercept, and slope of the regression line. The data should have a correlation coefficient not less than 0.995 (or coefficient of determination, $r^2 \geq 0.990$).This calculated value shall be within ±20% of the theoretical value. Deviations from these criteria will be addressed by reevaluating the calibration range, such that the calculated values meet these criteria.

7.4 Matrix Effects

Determination of LC-MS/MS matrix effects should be assessed as outlined in the analytical methods for both primary and confirmatory transitions in each soil matrix. Matrix effects should be evaluated for each test substance by fortifying a portion of control soil final extracts with fluometuron, des-methyl-fluometuron and CGA72903 and compared to a solvent-based calibration standard solution at the same concentration level. Matrix effects of >20% are considered to be significant. An evaluation of matrix effects would determine which type of calibration standards is necessary for analysis (matrix-matched vs. solvent standards).

8.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples, replicate analysis, and maintenance of material balance.

9.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

10.0 REPORTING

The raw data generated at Smithers Viscient will be peer-reviewed and the final report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The Quality Assurance Unit will inspect the final report to confirm that the methods, procedures, and observations are accurately and completely described, that the reported results accurately and completely reflect the raw data generated at Smithers Viscient and to confirm adherence with the study protocol. A single copy of the draft report will be submitted to the Sponsor for review. The report will be finalized according to Standard Operating Procedures. All reports will include, but will not be limited to, the following information:

- Protocol and all amendments.
- Name and address of study director and other contact person for ILV laboratory.
- Description of the analytical method.
- All recovery and control values for all matrices that were obtained during all ILV trials.
- Representative chromatograms/spectra for each analyte in each matrix.
- Description of the instruments used and operating parameters.
- Description of any problems encountered and a written description of any changes or modifications that were made during the ILV.

- Any steps considered critical, i.e. steps where little variation is allowable or directions must be followed precisely.
- The number of worker-hours required to complete one set of samples.
- The number of calendar days required for one set of samples.
- Any contact between the independent laboratory 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 confirmatory trial (i.e., after the first set, during the second set, etc.).
- The report and project numbers from Smithers Viscient and Sponsor study number (if any).
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program Coordinator (if applicable), Study Director and Principal Investigator.
- Identification of the test substance which may include chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, water solubility, vapor pressure, degree of purity of test substance (percent test chemical) (Sponsor-supplied, if available).
- The determined accuracy, precision, linearity, limit of detection, and method LOD.
- The mathematical equations and statistical methods used in generating and analyzing the data as well as calculations using these equations. Tabular and graphical representations (if appropriate) of the data.
- Description of any problems experienced and how they were resolved.
- Good Laboratory Practice (GLP) Compliance Statement signed by the Study Director.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.
- Location of the protocol, raw data and final report.

11.0 PROTOCOL AMENDMENTS

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor's contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any.

12.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide,