ABSTRACT

The purpose of this addendum to the study was per EPA's request to independently validate Valent USA Corporation's method RM-29S-1, *Determination of Flumiclorac Pentyl Ester and its Degradate, IMCA, in / on Soil* on an instrument that is different from the one that the method was first validated on. The method was successfully validated using soil.

Residues of flumiclorac pentyl ester and IMCA were extracted from each soil sample (2.5 grams) with 25 mL of an acetone/0.1 N HCl solution (4/1, v/v) by shaking for 10 minutes, centrifuging the sample and filtering the supernatant through glass wool into a 250 mL separatory funnel. The extraction was repeated by shaking each sample with a second 25-mL portion of acetone/0.1 N HCl (4/1, v/v) for 10 minutes. The sample was again centrifuged, and the supernatant filtered through the glass wool, combining the extracts in the separatory funnel. 5% Aqueous sodium chloride solution (75 mL) and dichloromethane (50 mL) were added to the sample, the sample was shaken for one minute, and the phases were allowed to separate. The lower dichloromethane layer was drained into a 250 mL round bottom flask by passing through about 50 g of sodium sulfate suspended over a plug of glass wool. Another portion of dichloromethane (50 mL) was added to the separatory funnel. The sample was shaken for one minute, the phases were allowed to separate, the dichloromethane layer was drained into the same 250 mL round bottom flask. The sodium sulfate cake was rinsed with dichloromethane (20 mL). The dichloromethane sample was evaporated to dryness using rotary evaporation, and the residue was redissolved in 5 mL of a 0.05% formic acid in methanol solution by sonicating briefly. This was transferred to a 10 mL volumetric flask. 3 mL of 0.05% formic acid in water was added to the 250 mL round bottom flask and briefly sonicated This was added to the same 10 mL volumetric flask, and diluted to volume with 0.05% formic acid in water. Prior to analysis the extract was diluted by a factor of 5. 200 µL of the extract was combined with 800 µL of 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This final solution was analyzed by liquid chromatography using a tandem mass spectrometer (LC/MS-MS). Samples were analyzed along with flumiclorac pentyl ester and IMCA standards of various concentrations, and five-point calibration curve (2^{nd} order polynomial fit, with weighting inversely proportional to the largest standard concentration) was used to quantify residues. The limits of quantitation (LOQ) for flumiclorac pentyl ester and IMCA are 0.02 ppm.

Two sample sets were extracted, and each set contained one reagent blank, one untreated soil sample and five fortified soil samples to facilitate sample handling in the laboratory.

EXPERIMENTAL

1.0 PROTOCOL AND METHOD

The protocol amendment number 1 for this independent laboratory validation is included in Appendix I. A copy of the method is included in the protocol.

2.0 MATERIALS

Lists of equipment, reagents and consumables used in the method validation are shown below. Similar equipment, reagents and consumables may also be used.

2.1 EQUIPMENT

Balance, analytical, capable of weighing 0.0001 g, Shimadzu
Balance, top loading, capable of weighing to 0.01 g, Mettler
Centrifuge, Beckman GPKR
Funnel, glass
Glass wool, borosilicate
Graduated cylinders, assorted volumes
Heated water bath, temperature ≤ 40°C
Pipettes, Pasteur
Pipettes, Volumetric; 5 mL and 10 mL
Pipettor, adjustable, capable of accurately dispensing volumes of up to 0.25 mL
Pipettor, automatic, capable of accurately dispensing volumes of up to 2.5 mL
Reciprocating mechanical shaker, Erbach
Rotary evaporator, Buchi Rotavapor-M
Round-bottom flasks, 250 mL
Separatory Funnels, 250 mL
Volumetric flasks, 10 mL, 50 mL, and 100 mL

2.2 REAGENTS AND CONSUMABLES

Acetone, HPLC grade, OmniSolv, EMD
Acetonitrile, HPLC grade, OmniSolv, EMD
Autosampler vials, 1.8 mL, with Teflon®-lined septum caps, E&K Scientific
Centrifuge tubes, 50 mL polypropylene with screw-caps, VWR
Formic Acid, reagent grade, 96+%, Sigma Aldrich
Methanol, HPLC grade, OmniSolv, EMD
Methylene Chloride, HPLC grade, OmniSolv, EMD
Sodium Chloride, reagent grade, JT Baker
Sodium Sulfate, residue grade, EMD
Water, HPLC grade, OmniSolv, EMD and deionized, in house

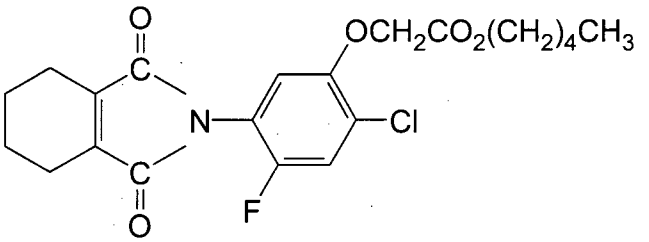
2.3 REAGENT SOLUTIONS

Acetone : 0.1 N HCl, 4:1 (v/v)
0.05% Formic Acid in HPLC grade water (HPLC mobile phase "A")
0.05% Formic Acid in Methanol (HPLC mobile phase "B")
0.05% Formic Acid in HPLC grade water : 0.05% Formic Acid in Methanol, 1:1 (v/v)
Sodium chloride solution, 5% w/v, in deionized water

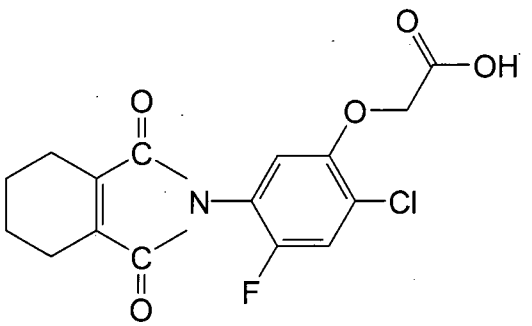
2.4 TEST SUBSTANCE / ANALYTICAL REFERENCE STANDARDS

The test substance/analytical reference standards were supplied by the Valent Technical Center in Dublin, CA. Copies of the certificate of quality are included in Appendix II.

Common name:	Flumiclorac-pentyl ester
Chemical name:	Pentyl[2-chloro-4-fluoro-5-(1,3,4,5,6,7-hexahydro-1,3-dioxo-2H-isoindol-2-yl)phenoxy]acetate
CAS number:	87546-18-7
Lot number:	AS 1675d
Stated purity:	98.9%
Expiration date:	November 1, 2013
Storage conditions:	Freezer
Structure:	



Common name:	IMCA
Chemical name:	2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido) phenoxyacetic acid
CAS number:	87547-04-4
Lot number:	AS 1780c
Stated purity:	99.3%
Expiration date:	December 18, 2014
Storage conditions:	Freezer
Structure:	



2.5 LC/MS-MS INSTRUMENTATION

Analyses were performed using a high pressure liquid chromatograph with a tandem mass spectrometer (LC/MS-MS), operating in positive ion mode. The instrument included the following components:

Agilent 1260 HPLC system comprised of a binary pump, autosampler and column oven
Applied Bioscience API 4000 #2 LC/MS-MS triple quadrapole mass spectrometer, with TurboSpray® electrospray ionization (ESI) sample introduction
Analyst® software (version 1.4.2)

2.6 TEST SYSTEM AND SAMPLE STORAGE

Untreated soil was obtained from the Valent Technical Center. The soil was stored at room temperature.

3.0 ANALYTICAL METHOD

3.1 PRINCIPLE OF THE METHOD

Flumiclorac pentyl ester and IMCA residues were extracted from the soil with an acetone/0.1 N HCl mixture (4/1, v/v), centrifuged to remove the solids, and filtered through glass wool into a separatory funnel. The extract was partitioned with dichloromethane after the addition of 5% aqueous sodium chloride solution. The dichloromethane was removed from the sample using rotary evaporation and the residues were dissolved in 0.05% formic acid in methanol and diluted to final volume with 0.05% formic acid in water. A portion of each sample was diluted by a factor of 5 into an autosampler vial for analysis.
Sample extracts and standards were analyzed using a high pressure liquid chromatograph with a tandem mass spectrometer (LC/MS-MS). A five-point, 2nd order polynomial calibration curve (weighted relative to the largest standard concentration) was used to quantify flumiclorac pentyl ester and IMCA in the sample extracts.

3.2 LIMITS OF QUANTITATION (LOQ)

The LOQ in soil was 0.02 ppm (µg/g) for both flumiclorac pentyl ester and IMCA.

3.3 PREPARATION OF STANDARD STOCK SOLUTIONS

3.3.1 1,000 µg/mL standard stock solutions

Approximately 50 mg of flumiclorac pentyl ester was weighed (taking into account the purity of the standard) and brought to volume in a 50 mL volumetric flask with acetone. This 1,000 µg/mL standard solution was stored in a refrigerator when not in use.

Approximately 50 mg of IMCA was weighed (taking into account the purity of the standard) and brought to volume in a 50 mL volumetric flask with acetone. This 1,000 µg/mL standard solution was stored in a refrigerator when not in use.

3.3.2 10 µg/mL fortification solution

A 10 µg/mL fortification solution was prepared by transferring 1.0 mL of the 1,000 µg/mL flumiclorac pentyl ester standard stock solution and 1.0 mL of the 1,000 µg/mL IMCA standard stock solution into a 100 mL volumetric flask and filling the flask to the mark with acetone. This standard solution was stored in a refrigerator when not in use.

3.3.3 1 µg/mL fortification solution

A 1.0 µg/mL fortification solution was prepared by transferring a 10.0 mL aliquot of the 10 µg/mL fortification solution into a 100 mL volumetric flask and filling the flask to the mark with acetone. This standard solution was stored in a refrigerator when not in use.

3.3.4 0.05 µg/mL linearity solution

A 0.05 µg/mL linearity solution was prepared by transferring a 2.5 mL aliquot of the 1.0 µg/mL fortification solution into a 50 mL volumetric flask and filling the flask to the mark with 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This standard solution was stored in a refrigerator when not in use.

3.3.5 0.01 µg/mL linearity solution

A 0.01 µg/mL linearity solution was prepared by transferring a 1.0 mL aliquot of the 1.0 µg/mL fortification solution into a 100 mL volumetric flask and filling the flask to the mark with 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This standard solution was stored in a refrigerator when not in use.

3.3.6 0.005 µg/mL linearity / calibration solution

A 0.005 µg/mL linearity solution was prepared by transferring a 5.0 mL aliquot of the 0.05 µg/mL linearity solution into a 50 mL volumetric flask and filling the flask to the mark with 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This standard solution was stored in a refrigerator when not in use.

3.3.7 0.001 µg/mL linearity solution

A 0.001 µg/mL linearity solution was prepared by transferring a 10.0 mL aliquot of the 0.005 µg/mL linearity solution into a 50 mL volumetric flask and filling the flask to the mark with 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This standard solution was stored in a refrigerator when not in use.

3.3.8 0.0005 µg/mL linearity solution

A 0.0005 µg/mL linearity solution was prepared by transferring a 5.0 mL aliquot of the 0.005 µg/mL linearity solution into a 50 mL volumetric flask and filling the flask to the mark with 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v). This standard solution was stored in a refrigerator when not in use.

3.4 PREPARATION OF SAMPLES AND WEIGHING

2.5 g subsamples of the soil sample were weighed into 50-mL centrifuge tubes for extraction.

3.5 PREPARATION OF FORTIFICATION SAMPLES

For the LOQ fortifications (0.02 ppm), each 2.5 g soil sample was fortified with 50 µL of the 1.0 µg/mL fortification solution. For the 0.20 ppm fortifications, 50 µL of the 10.0 µg/mL fortification solution was added to the 2.5 g soil samples.

3.6 EXTRACTION PROCEDURE

3.6.1 Extraction with acetone/0.1 N HCl

A 25-mL portion of acetone : 0.1 N HCl (4:1, v/v) was added to each sample, the tubes were capped, and the samples were shaken on a mechanical shaker for approximately 10 minutes. The samples were centrifuged at ~2000 rpm for about 5 minutes, and the supernatant was decanted through a glass wool plug into a 250 mL separatory funnel. The sample was extracted a second time with an additional 25 mL of acetone : 0.1 N HCl (4:1, v/v) by shaking the sample for about 10 minutes, centrifuging the samples and decanting the supernatant through the glass wool into the same 250 mL separatory funnel, combining the two extracts.

3.6.2 Dichloromethane/Water partition

A 75 mL aliquot of the 5% aqueous NaCl solution was added to the 250 mL separatory funnel, and 50 mL of dichloromethane was added. The sample was shaken for approximately 1 minute, the phases allowed to separate, and the lower dichloromethane layer was drained into a 250 mL round bottom flask over a cake of sodium sulfate suspended over a plug of glass wool in a glass funnel. Another portion of 50 mL dichloromethane was added, and the sample was partitioned a second time. The lower dichloromethane layer was drained into the same 250 mL round-bottom flask. The dichloromethane sample was evaporated to dryness using a rotary evaporator with a water bath at ≤40 °C and the residues were redissolved in 5 mL of 0.05% formic acid in methanol.

3.6.3 Final Volume

The 250 mL round bottom flask was sonicated briefly and sample was transferred to a 10 mL volumetric flask. 3 mL of 0.05% formic acid in water was added to the 250 mL round bottom flask and sonicated. This was also transferred into the 10 mL volumetric flask. This extract was diluted to volume with 0.05% formic acid in water. To vial for analysis, 200 µL of the extract was combined with 800 µL of 0.05% formic acid in methanol : 0.05% formic acid in water (1:1, v/v).

3.7 LC/MS-MS OPERATION PARAMETERS

3.7.1 HPLC Conditions

Column: YMC ODS-AM, 3 micron, 100 x 3 mm
Mobile phases: 0.05% formic acid (FA) in HPLC water
0.05% formic acid (FA) in methanol

Flow rate: 500 µL/min.

Gradient program:

Time (min.)	% 0.05% formic acid in Water	% 0.05% formic acid in Methanol
0.0	50	50
1.0	50	50
6.0	10	90
10.0	10	90
10.5	50	50
15.0	50	50

Retention times:

IMCA	7.44 min
Flumiclorac pentyl ester	9.28 min

Injection volume: 25 µL

3.7.2 MS/MS Conditions

The mass spectrometer parameters were optimized for flumiclorac pentyl ester and IMCA prior to the sample analysis. Operating parameters used during the method validation were:

Scan Type: MRM
Polarity: Positive
Ion Source: TurboSpray® electro spray interface
Q1 and Q3 Resolution: unit

Transition Parameters:

	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Lenses (DP)	(EP)	(CE)	(CXP)
Flumiclorac-pentyl ester	424.0	308.1	150	55	15	25	20
IMCA	354.0	308.1	150	50	12	20	4

3.7.3 Analytical Sequence Setup

For each set of analyses, the LC-MS/MS instrument was conditioned with several injections of a soil matrix sample before the injection of the first continuing standard. The analytical sequence then began with the continuing standard injection (a mid-level instrument calibration standard), then one or two samples followed by a calibration standard, etc., and ended with a continuing standard injection. The continuing standard was also injected at least once within the middle of the sequence.

3.7.4 Data Integration

The Analyst software associated with the instrument was used to integrate the peaks of interest, and report the area count responses for each standard and sample injection. The integrations were based on the mass transitions of 424.0 → 308.1 mz and 354.0 → 308.1 mz.

3.8 CALCULATIONS

3.8.1 Calibration procedures

The area response data provided by the Analyst software was entered into an Excel® spreadsheet to perform the calculations. A weighted 2nd order polynomial fit curve was generated for each set of analyses, and the curve constants (a, b and c) were determined. The curve was used to calibrate the instrument, determine the acceptability of the standard injections and to calculate the sample residues. The curve was generated by plotting the standard responses versus the standard concentration, and was weighted relative to the largest standard concentration, as shown below:

Standard Concentration	Number of times used for weighting
0.0005	100
0.001	50
0.005	10
0.010	5
0.05	1

3.8.2 Calculation of analyte concentrations and sample residues

Analyte concentrations for the standards and the samples were calculated by the Excel spreadsheet using the equation:

$$Y = (aX^2 + bX + c) = \mu g/mL$$

where:
- Y = concentration of analyte, µg/mL
- X = peak area
- a = calibration curve coefficient
- b = calibration curve coefficient
- c = calibration curve coefficient (intercept)

and the sample residues, as ppm, were calculated using the formula:

$$\frac{\mu g/mL \times (\text{Final Sample Vol., mL}) \times (\text{Dilution Factor})}{\text{Sample Wt, g}} = \text{ppm } (\mu g/g)$$

Example calculation:
The concentration of IMCA in sample Ft 1, fortified at 0.02 ppm, analyzed on June 21, 2013 is:

area in UTC = 0
area in Ft 1 = 14300
X = 14300 - 0
a = 4.41×10^{-13}
b = 6.90×10^{-5}
c = -3.34×10^{-2}
Sample Weight (crop equivalent) = 2.5 g
Final Sample Volume = 10 mL
Dilution Factor = 5

$$Y = [(4.41\times10^{-13}\times14300^2)+(6.90\times10^{-5}\times14300)+(-3.34\times10^{-2}] = 0.0009534 \text{ µg/mL}$$

and

$$\text{ppm} = \frac{0.0009534 \times 10 \times 5}{2.5} = 0.0191 \text{ ppm}$$

3.8.3 Calculation of fortification sample percent recovery

To calculate the percent recoveries for the fortified soil samples, the ppm residues found in the fortified samples were divided the by the fortification level.

Example calculation:
Percent recovery for sample Ft 1 extracted and analyzed on June 21, 2013:

0.0191 ppm ÷ 0.02 ppm fortification level × 100% = 95.3%

Note: these calculations, when done by hand, may differ slightly from the results reported by the Excel spreadsheet due to rounding differences.

3.8.4 Calculation of standard deviation and coefficient of variation

Standard deviations and Coefficient of Variations (CV) were used to evaluate the data for the standard injections and for the fortification samples. The standard deviations were calculated from the mean values of the samples being considered, and expressed as an absolute percent value (Coefficent of Variation, CV) using the mean values:

$$\text{Coefficient of Variation, \%} = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

3.8.5 Acceptance Criteria

The criteria for the acceptance of an analytical set was 1) the standard regression curve was required to be at least 0.99, 2) the calculated standard concentration for each standard injection was required to be within 15% of the nominal concentration and 3) the coefficient of variation (CV) for the continuing standard injection responses was required to be <10%.

3.8.6 Threshold area counts

Using a 25 µL injection, the signal to noise ratio for flumiclorac pentyl ester was approximately 30:1 for the smallest standard injection. Minor response of flumiclorac pentyl ester was observed in one reagent blank analysis, however the response was less than 15% of the smallest standard and was therefore not considered to be flumiclorac pentyl ester and was reported as "0 ppm". Fortification recoveries were adjusted for the responses in the control samples by subtracting the area count response of the untreated control sample from the area response of each fortified sample.

3.9 STATISTICS STATEMENT

The mean percent recoveries, standard deviations, and coefficients of variation were calculated using Excel spreadsheets. The Excel spreadsheets also calculated the 2nd order polynomial calibration curves (weighted relative to the largest standard concentration) and the coefficient of determination (r^2) for each of the standard calibration curves.

4.0 DEVIATIONS

None.