INTRODUCTION

This study was conducted following procedure described in the EPA Ecological Effects Test Guidelines, OPPTS 850.7100: Data Reporting for Environmental Chemistry Methods, (d) Independent Laboratory Validation. This study was initiated on January 4, 2012 the day the Study Director signed the protocol (Appendix 1). Per EPA's request, the study was repeated on a different instrument than that used in the Valent's method RM-29W-4 (Appendix 2). The laboratory work was conducted on January 4 – January 12, 2012 (original report) and May 24, 2013 – June 13, 2013 (addendum). The study was conducted at Valent Technical Center, located in Dublin, California, U.S.A.

Quality assurance measures taken during the experimental portion of this study included, but were not limited to, the following:

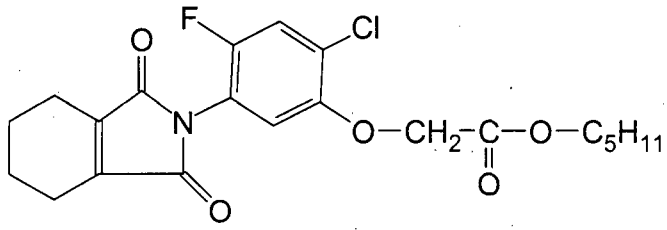
- All measuring equipment used in this study was calibrated in accordance with the proper Valent Standard Operating Procedures.
- The original protocol, all raw data, and the final report for this study are stored in the Valent archive located at the Valent Technical Center, Dublin, California.
- An in-life audit and audit of the data and the report were performed by the Valent QA unit.
- A subsample of the test/reference substances used in this study was archived at the Valent Technical Center, Dublin, California.

MATERIALS

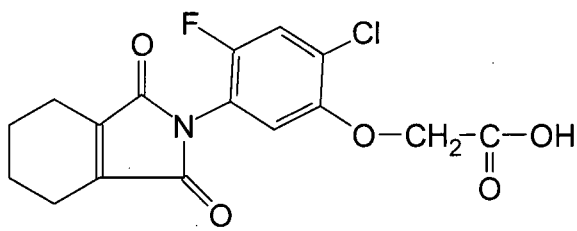
Test /reference substances:

The test/reference substances were supplied by the Valent Technical Center in Dublin, CA. Copies of the certificate of quality for the test/reference substances are included in Appendix 3.

Common Name:	Flumiclorac-pentyl ester
Chemical Name (CA):	Acetic acid, 2-[2-chloro-4-fluoro-5-(1,3,4,5,6,7-hexahydro-1,3-dioxo-2H-isoindol-2-yl)phenoxy]-,pentyl ester
Other Name:	Pentyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophtalimido)phenoxyacetate
CAS Number:	87546-18-7
Molecular Weight:	423.86
Molecular Formula:	$C_{21}H_{23}ClFNO_5$
Lot Number:	AS 1675d
Chemical Purity:	98.9%
Expiration Date:	Nov. 1, 2013
Chemical Structure:	



Common Name:	IMCA
Chemical Name (CA):	Acetic acid, 2-[2-chloro-4-fluoro-5-(1,3,4,5,6,7-hexahydro-1,3-dioxo-2H-isoindol-2-yl)phenoxy]-
Other Name:	2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophtalimido)phenoxyacetate acid
CAS Number:	87547-04-4
Molecular Weight:	353.73
Molecular Formula:	$C_{16}H_{13}ClFNO_5$
Lot Number:	AS 1780c
Chemical Purity:	99.3%
Expiration Date:	Dec. 18, 2013
Chemical Structure:	



Test system:

The test system was Dublin, CA city tap water fortified with the test substances.

Chemicals and Reagents:

Methanol suitable for GC, HPLC, and Spectrophotometry (EMD Chemicals, Gibbstown, NJ, U.S.A.);

Water suitable for HPLC, Spectrophotometry, Gas Chromatography, and Gradient Analysis (EMD Chemicals, Gibbstown, NJ, U.S.A.);

Ethyl Acetate suitable for GC, HPLC, and Spectrophotometry (EMD Chemicals, Gibbstown, NJ, U.S.A.);

Acetone suitable for GC, HPLC, and Spectrophotometry (EMD Chemicals, Gibbstown, NJ, U.S.A.);

Hydrochloric Acid, 36.5-38% (Mallinckrodt Baker, Inc., Phillipsburg, NJ, U.S.A.);

Formic Acid, LC/MS grade (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Reagent solutions:

HPLC-grade water acidified with 0.05% formic acid;

Methanol acidified with 0.05% formic acid;

Methanol/HPLC-grade water (1:1, v/v) acidified with 0.05% formic acid;

0.1 N Hydrochloric Acid.

Glassware:

A list of glassware used in the method validation is shown below. Similar glassware from other suppliers may also be used.

100 and 10 mL volumetric flasks (Fisher Scientific, Pittsburgh, PA, U.S.A.);

Volumetric pipettes, variable volumes (Fisher Scientific, Pittsburgh, PA, U.S.A.);

Disposable Pasteur pipettes, 150-mm long, 5 mm I.D. (VWR, Radnor, PA, U.S.A.);

Centrifuge tubes, polypropylene free standing with flat caps, 50 mL (VWR, Radnor, PA, U.S.A.);

Round bottom flasks with ground glass joint, size 24/40, 50 mL (Fisher Scientific, Pittsburgh, PA, U.S.A.);

Glass vials, screw-cap, 20 mL (Fisher Scientific, Pittsburgh, PA, U.S.A.);
Autosampler vials, 1.8 mL, with Teflon®-lined septum caps (Fisher Scientific, Pittsburgh, PA,

U.S.A.).

Equipment and supplies:

A list of equipment used in the method validation is shown below. Similar equipment from other suppliers may also be used.

Balance, Mettler-Toledo XS204 (Mettler-Toledo Inc., Columbus, OH, U.S.A.);

Hamilton microsyringes, 100 µL, 500 µL (Fisher Scientific, Pittsburgh, PA, U.S.A.);

Vacuum rotary evaporator equipped with a temperature controlled water bath;

Ultrasonic cleaner, Branson 3510 (Branson Ultrasonics Corp., Danbury, CT, U.S.A.);

Rainin autopipettes, 250 µL and 1000 µL (Rainin Instrument LLC, Oakland, CA, U.S.A.);

YMC Pack ODS-AM column, S-3 µm, 120 nm, 3.0×100 mm (YMC America Inc., Allentown, PA, U.S.A.);

Agilent 1260 Infinity HPLC system equipped with a column thermostat compartment, a binary pump, a degasser, and an autosampler (Agilent Technologies Inc., Palo Alto, CA, U.S.A.);

Applied Bioscience API 4000 triple quadrupole mass spectrometer operating in positive electron spray ionization mode (ESI) (Applied Biosystems, Foster City, CA);

Analyst® software (version 1.4.2) (Applied Biosystems, Foster City, CA).

ANALYTICAL METHOD

Validation sample sets:

One trial was conducted with a validation set consisted at least of instrument calibration standards, one reagent blank, two unspiked control samples, five water samples fortified with analytes at 2.0 µg L^{-1} (LOQ), and five water samples fortified with analytes at 20.0 µg L^{-1} (10XLOQ).

Preparation of standard stock solutions:

Stock solution of flumiclorac-pentyl ester (1.00 mg mL^{-1}). A flumiclorac-pentyl ester stock solution (1.00 mg mL^{-1}) was prepared by accurately weighing 101.3 mg (100.2 mg a.i.) of the flumiclorac-pentyl ester standard into a 100 mL volumetric flask and adding acetone to the mark. The concentration of the standard was corrected for the purity of test/reference substance.

Stock solution of IMCA (1.00 mg mL^{-1}). An IMCA stock solution (1.00 mg mL^{-1}) was prepared by accurately weighing 100.8 mg (100.1 mg a.i.) of the IMCA standard into a 100 mL volumetric flask and adding acetone to the mark. The concentration of the standard was corrected for the purity of test/reference substance.

Fortifying standard solution (10.0 µg mL^{-1}). A fortifying standard solution containing both flumiclorac-pentyl ester and IMCA (10.0 µg mL^{-1}) was prepared by diluting the stock solutions of flumiclorac-pentyl ester and IMCA in acetone. For that, 1 mL of each stock solution of flumiclorac-pentyl ester and IMCA (1.00 mg mL^{-1}) was transferred to a 100 mL volumetric flask and diluted to the mark with acetone. This solution was used to fortify water samples at the 20.0 ng mL^{-1} level.

Fortifying standard solution (1.00 µg mL^{-1}). A fortifying standard solution containing both flumiclorac-pentyl ester and IMCA (1.00 µg mL^{-1}) was prepared by diluting the fortifying solution containing flumiclorac-pentyl ester and IMCA. For that, 10 mL of the fortifying solution of flumiclorac-pentyl ester and IMCA (10.0 µg mL^{-1}) was transferred to a 100 mL volumetric flask and diluted to the mark with acetone. This solution was used to fortify water samples at the 2.00 ng mL^{-1} level.

All stock and fortifying solutions of the test/reference substances were stored in a refrigerator (~4 °C) when not in use.

Preparation of instrument calibration and continuing standard solutions:

Instrument calibration standard solution, 50.0 ng mL^{-1}. A 50.0 ng mL^{-1} instrument calibration standard solution was prepared by transferring 5 mL of the 1.00 µg mL^{-1} fortification standard solution containing both flumiclorac-pentyl ester and IMCA into a 100 mL volumetric flask and diluting the flask to the mark with methanol/HPLC-grade water acidified with 0.05% formic acid (1/1, v/v). This standard solution was stored in a refrigerator when not in use.

Instrument calibration standard solution, 10.0 ng mL^{-1}. A 10.0 ng mL^{-1} instrument calibration standard solution was prepared by transferring 2 mL of the 50.0 ng mL^{-1} instrument calibration standard solution containing both flumiclorac-pentyl ester and IMCA into a 10 mL volumetric flask and diluting the flask to the mark with methanol/HPLC-grade water acidified with 0.05% formic acid (1/1, v/v). This standard solution was stored in a refrigerator when not in use.

Instrument calibration standard solution, 5.00 ng mL^{-1}. A 5.00 ng mL^{-1} instrument calibration standard solution was prepared by transferring 1 mL of the 50.0 ng mL^{-1} instrument calibration standard solution containing both flumiclorac-pentyl ester and IMCA into a 10 mL volumetric flask and diluting the flask to the mark with methanol/HPLC-grade water acidified with 0.05% formic acid (1/1, v/v). The 5.00 ng mL^{-1} instrument calibration standard solution was used as a continuing calibration standard solution. This standard solution was stored in a refrigerator when not in use.

Instrument calibration standard solution, 1.00 ng mL^{-1}. A 1.00 ng mL^{-1} instrument calibration standard solution was prepared by transferring 0.2 mL of the 50.0 ng mL^{-1} instrument calibration standard solution containing both flumiclorac-pentyl ester and IMCA into a 10 mL volumetric flask and diluting the flask to the mark with methanol/HPLC-grade water acidified with 0.05% formic acid (1/1, v/v). This standard solution was stored in a refrigerator when not in use.

Instrument calibration standard solution, 0.50 ng mL^{-1}. A 0.50 ng mL^{-1} instrument calibration standard solution was prepared by transferring 0.1 mL of the 50.0 ng mL^{-1} instrument calibration standard solution containing both flumiclorac-pentyl ester and IMCA into a 10 mL volumetric flask and diluting the flask to the mark with methanol/HPLC-grade water acidified with 0.05% formic acid (1/1, v/v). This standard solution was stored in a refrigerator when not in use.

Preparation of fortification samples:

LOQ fortification samples at 2.00 µg L^{-1} (2.00 ng mL^{-1}; or 2.00 ppb) were prepared by adding 40 µL of the 1.00 µg mL^{-1} fortifying standard solution containing both flumiclorac-pentyl ester and IMCA into each of five 50 mL polypropylene centrifuge tubes containing 20 mL of Dublin tap water using a 100 µL Hamilton microsyringe.

10XLOQ fortification samples at 20.0 µg L^{-1} (20.0 ng mL^{-1}; or 20.0 ppb) were prepared by adding 40 µL of the 10.0 µg mL^{-1} fortifying standard solution containing both flumiclorac-pentyl ester and IMCA into each of five 50 mL polypropylene centrifuge tubes containing 20 mL of Dublin tap water using a 100 µL Hamilton microsyringe.

Extraction procedure:

Water samples were extracted in one batch of thirteen samples (1 reagent blank, 2 unspiked control samples, and 10 water samples fortified with the analytes at 2.00 µg L^{-1} or 20.0 µg L^{-1}). Before extraction, 1 mL of 0.1 N Hydrochloric acid was added to sample, and the sample was shaken briefly. Next, 10 mL of ethyl acetate was added to the sample, the centrifuge tube containing the sample was capped and shaken for 1 min. The layers were allowed to separate and the upper layer of ethyl acetate was pipetted into a 50 mL round-bottom flask. A fresh portion of ethyl acetate, 10 mL, was added to the sample and extraction was repeated. The ethyl acetate extracts were combined into the 50 mL round-bottom flask, and then ethyl acetate was removed using a vacuum rotary evaporator equipped with a water bath less than 40 °C. After concentration, the residues were first re-dissolved in 5 mL of methanol acidified with 0.05% formic acid, and the sample was sonicated for about 15 sec. The methanol extract was transferred into a 10 mL volumetric flask. Then approximately 3 mL of HPLC-grade water acidified with 0.05% formic acid was added to the round-bottom flask, sonicated for about 15 sec, and the water extract was combined with methanol extract in the 10 mL volumetric flask. The extract was adjusted to the final volume of 10 mL using HPLC-grade water acidified with 0.05% formic acid. The prepared extract was diluted 1:4 (250 µL of the sample and 750 µL of methanol/HPLC-grade water acidified with 0.05% formic acid, 1:1, v/v) in an autosampler vial (1.8 mL) and analyzed by a liquid chromatography/mass spectrometry (LC/MS/MS).

LC/MS/MS operation parameters:

HPLC operation conditions. Operating parameters used during the independent method validation were:

Analytical HPLC Column:	YMC-ODS-AM column, 100×3.0 mm, S-3µm, 120Å
Mobile Phase:	Methanol acidified with 0.05% formic acid
	HPLC-grade water acidified with 0.05% formic acid

Gradient Program:

Time (min)	Flow Rate ($\mu L\ min^{-1}$)	%Water with 0.05% Formic Acid	%Methanol with 0.05% Formic Acid
0.00	500	50	50
1.00	500	50	50
6.00	500	10	90
10.0	500	10	90
10.5	500	50	50
15.0	500	50	50

Flow Rate:	500 $\mu L\ min^{-1}$	
Column Temperature:	30 °C	
Injection Volume:	25 µL	
Run Time:	15 min	
Retention times (approximate):	Flumiclorac-pentyl ester	9.3 min
	IMCA	7.3 min

Divert Valve Position Program:

Time, min	Valve Position
0.00	waste
5.00	MS/MS
10.00	waste
15.00	waste

LC/MS/MS conditions. The mass spectrometer parameters were optimized for the flumiclorac-pentyl ester and IMCA analytes prior to the sample analysis. Operating parameters used during the independent method validation were:

	Flumiclorac-pentyl ester	**IMCA**
Scan Type	MRM	MRM
Polarity	Positive	Positive
Ion Source	TurboSpray® electro spray interface	
Period, (min)	8-15	0-8
Dwell time, (msec)	150	150
Q1/Q3 mass, (amu)	424.1/308.0	354.0/308.1
Declustering potential, (V)	80	80
Entrance potential, (eV)	10	10
Collision cell exit potential, (V)	9	9
Collision energy, (V)	30	25
Collision activated dissociation gas, (psig)	6	6
Curtain gas, (psig)	25	20
Ion source gas 1, nebulizer gas, (psig)	40	40
Ion source gas 2, heater gas, (psig)	20	45
Ion spray voltage, (V)	5500	5500
Temperature, (°C)	500	600

Analytical Sequence Setup:

For each set of analyses, the LC/MS/MS instrument was conditioned with several injections of a water sample extract before the injection of the first continuing calibration standard. The analytical sequence then began with the continuing standard injection (a mid-level instrument calibration standard), then one or two samples followed by an instrument calibration standard, etc., and ended with a continuing standard injection. The continuing standard was also injected at least several times through the sequence. A single injection was performed for each instrument calibration and continuing standard solutions and samples.

Data Integration:

The Analyst software associated with the instrument was used to integrate the peaks of interest. The integration was based on the Extracted Ion Chromatogram (XIC for the analyte).

Calculations:

Calibration procedures. The flumiclorac-pentyl ester and IMCA analytes were calibrated externally using a 2nd order polynomial regression with 1/concentration weighting. The peak area (detector response) data provided by the Analyst software was entered into a GraphPad Prism 4.03 Software (GraphPad Software Inc.). A 2nd order polynomial fit curve ($Y=a+bX+cX^2$) with 1/concentration weighting was generated for the analytes with 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 instrument calibration standard peak area (detector responses, X) versus the instrument calibration standard concentration (Y).

Calculation of analyte concentrations and sample residues. Analyte concentrations for the standards and the samples were calculated by the Excel spreadsheet using the equations 1 and 2, respectively:

$$Y = a + bX + cX^2 \quad (1)$$

$$Y = \frac{(a + bX + cX^2) \times FV \times DF}{SV} \quad (2)$$

where X – peak area (detector response),
Y – concentration of analyte, ng mL^{-1} (µg L^{-1}, ppb),
a – calibration curve coefficient,
b – calibration curve coefficient,
c – calibration curve coefficient,
FV – final sample volume, mL,
DF – dilution factor,
SV – sample volume, mL.

Example calculation:
Concentration of flumiclorac-pentyl ester in the sample LOQ A, 2.00 ng mL^{-1}, extracted 31-May-2013 and analyzed on 31-May-2013:
X = 35100,
a = -5.458×10^{-3}

$b = 2.384\times10^{-5}$,
$c = 3.854\times10^{-13}$,
Final Sample Volume = 10 mL,
Dilution factor = 4
Sample Volume = 20 mL

$$Y = [-5.458\times10^{-3} + 2.384\times10^{-5}\times35100 + 3.854\times10^{-13}\times(35100)^2]\times10\times4\div20 = 1.66\ \text{ng mL}^{-1}$$

Calculation of fortification sample percent recovery. To calculate the percent recoveries for the fortified water samples, ng mL^{-1} (ppb), residues found in the control samples (if any) was subtracted from the ng mL^{-1} (ppb) residues found in the fortified samples, and then dividing the result by the fortification level.

Example calculation:
Percent recovery for sample LOQ A extracted 31-May-2013 and analyzed on 31-May-2013:

(1.66 ng mL^{-1} in fortified – 0.00 ng mL^{-1} in unspiked) ÷ 2.00 ng mL^{-1} fortification level × 100% =

= 1.66 ÷ 2.00 × 100% = 83.0%

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

Calculation of standard deviation and coefficient of variation. Standard deviations (SD) and or coefficient of variations (CV) were used to evaluate the data for the fortification samples. The coefficients of variations (CV) were calculated from the mean values of the samples being considered, and expressed as an absolute percent:

$$\text{Coefficient of Variation, \%} = \frac{\text{Standard Deviation}}{\text{Mean}}\times100$$

Example calculation:

Using the five replicates fortified at 2.00 ng mL^{-1} (LOQ) in the analytical set, flumiclorac-pentyl ester, fortification recoveries, where the mean recovery was 85.8% and the standard deviation was 2.1%:

$$\text{CV, \%} = \frac{2.1\%}{85.8\%}\times100\% = 2.4\%$$

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

Acceptance Criteria. The criteria for the acceptance of an analytical set was

1) the coefficient of determination (r^2) of the standard regression curve was required to be ≥0.99;
2) the calculated standard concentration for each standard injection was required to be within 15% of the nominal concentration;
3) the coefficient of variation (CV) for the continuing standard injection responses was required to be ≤15%.

Threshold area counts. Using a 25 µL injection, the signal-to-noise ratio for flumiclorac-pentyl

ester and IMCA for the smallest standard (0.50 ng mL^{-1}) was about 104:1 and 28:1, respectively.

Statistics statement:

The average percent recoveries, standard deviations, and coefficients of variation were calculated using Excel spreadsheets. The GraphPad Prism 4.03 Software calculated the 2nd order polynomial fit curve ($Y=a+bX+cX^2$) and the coefficient of determination (r^2) for each of the standard calibration curves.