1.0 EXECUTIVE SUMMARY

North Coast Laboratories, Ltd. (NCL) performed an independent laboratory validation (ILV) of method RM-49S-1, Ethaboxam: Determination of Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 in Soil as requested by Valent U.S.A Corporation. This study was designed to fulfil the requirements of EPA's Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2].

Ethaboxam residues were determined in soil samples by extracting the sample with acetone/water (3:1, v/v) and then centrifuging the extracts. The sample volume was adjusted with methanol and then diluted with high performance liquid chromatography (HPLC)-grade water. The extract was filtered and then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in both the positive and negative ion modes. An Applied Biosystems/MDS Sciex API 4000™ mass spectrometer with an ACE 5 C18 analytical column was used for the analysis. The instrument was calibrated using The Analyst® Software (version 1.6).

The analytical validation set consisted of a reagent blank (taken through the method), duplicate untreated control (UTC) soil samples, five UTC soil samples fortified at 0.010 µg/g the limit of quantitation (LOQ) of the method and five UTC water samples fortified at 0.10 µg/g (ten times the LOQ of the method).

2.0 INTRODUCTION

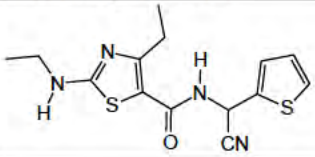
This report describes the independent laboratory validation (ILV) of the analytical method RM-49S-1, “Ethaboxam: Determination of Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 in Soil” [3] as performed by North Coast Laboratories, Ltd. (NCL).

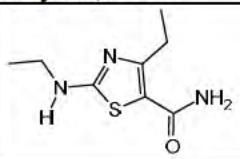
This study fulfils the requirements of EPA's Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2].

3.0 MATERIALS AND METHODS

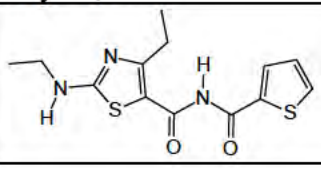
3.1 Test Substance

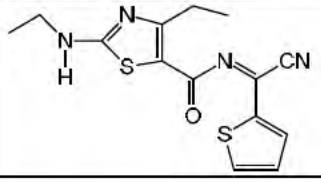
The test substance and standards were shipped from Valent U.S.A. Corporation, Dublin, CA to NCL. They were received on December 17, 2014, with the exception of *d5*-ethaboxam which was received December 19, 2014. The test substance/standards that were used for the validation are described as follows:

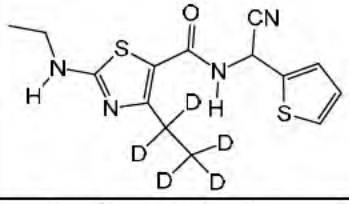
Common Name	Ethaboxam
Company Experimental Name	V-10208
IUPAC Name	*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide
Source	Valent Technical Center
Purity	100 % (wt/wt)
Lot Number	AS 2293a
Other Identification	VT-1269-21
Expiration Date	February 3, 2016
Structural Formula	

Company Experimental Name	LGC-32523
IUPAC Name	4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide
Source	Valent Technical Center
Purity	97.5 % (wt/wt)
Lot Number	AS 2313b
Other Identification	14SC8415162
Expiration Date	July 23, 2016
Structural Formula	

Company Experimental Name	LGC-32533
IUPAC Name	4-ethyl-2-(ethylamino)-*N*-(thiophen-2-ylcarbonyl)-1,3-thiazole-5-carboxamide
Source	Valent Technical Center
Purity	99.9 % (wt/wt)
Lot Number	AS 2315b

Other Identification	14SC8387971
Expiration Date	July 24, 2016
Structural Formula	

Company Experimental Name	LGC-32799
IUPAC Name	*N*-[(*Z*)-cyano(thiophen-2-yl)methylidene]-4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide
Source	Valent Technical Center
Purity	97.8 % (wt/wt)
Lot Number	AS 2410a
Other Identification	14SC838794
Expiration Date	October 6, 2015
Structural Formula	

Company Experimental Name	*d5*-Ethaboxam
IUPAC Name	*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide-d5
Source	Valent Technical Center
Purity	98% (wt/wt)
Lot Number	13-SDJ-181-1
Other Identification	
Expiration Date	October 23, 2016
Structural Formula	

Valent U.S.A. Corporation, Dublin, CA maintains the characterization and stability data for the test substance.

Stock standard solutions were prepared from the neat test substance/standards for use in the preparation of fortification solutions and instrument calibration solutions. All standard solutions were as per the method except at different concentrations. The stock standards were stored refrigerated when not in use. Section 3.5.4 describes the preparation of the stock solutions, and Section 4.7.2 provides example calculations.

3.2 Equipment and Reagents

3.2.1 Solvents and Reagents

HPLC Water (Fisher)
Acetone, pesticide grade (Fisher)
Ammonium acetate (Aldrich)
Methanol HPLC grade (Fisher)
Formic acid (Veritas)

3.2.2 Apparatus

A list of apparatus used in the method validation trial is shown below.

Mettler AB204-2 Analytical Balance
Volumetric flasks of assorted volumes
Assorted syringes
Assorted automatic pipettes
50 mL graduated centrifuge tubes
Centrifuge
Glass bottles, assorted sizes
Syringe, 3 mL polypropylene
Syringe filter Teflon 0.2 micron
Screw thread amber 15-mL glass vials with Teflon-lined screw-caps
1.8-mL clear screw top standard mouth glass autosampler vials with caps
Reciprocating mechanical shaker

3.2.3 LC-MS/MS Instrumentation

Analysis was performed using a (LC-MS/MS). The following equipment was used:

LC/MS-MS system-

Shimadzu model LC-10 AD vp, pumps (two each)

Shimadzu model SLC-10 A vp, pump controller (one each)

Perkin Elmer Series 200 autosampler

Phenomenex six-port, two position, switching valve for diverting column effluent to waste.

Applied Biosystems/MDS Sciex API 4000™ mass spectrometer with Turbo V® pneumatically assisted electrospray ionization interface

Two Zorbax Eclipse XDB-C18, 4.6 x 50 mm, 5-micron analytical columns. One column is for the positive ion analytes and the second is used for the negative ion analyte.

3.3 Safety and Health

This method was performed by trained personnel who acted in accordance with the material safety data sheet (Appendix 4) that documents the hazards associated with the use of this chemical.

3.4 Test System and Sample Storage

The soil sample V-38327CA Bulk Soil sent from Valent was used as the matrix for the validation. The soil sample was received on December 17, 2014. It was given a unique North Coast Laboratories, Ltd. (NCL) Client Sample ID of 1412366-01A. The sample was stored frozen upon receipt.

3.5 Analytical Method and Method Establishment

3.5.1 Principle of the Method

The sample preparation involved extracting the soil sample with acetone/water (3:1, v/v) and then centrifuging the extracts. The sample volume was adjusted with methanol and then diluted with HPLC-grade water. The extract was filtered and then analyzed by LC-MS/MS in both the positive and negative ion modes.

3.5.2 Limits of Quantitation

The LOQ for ethaboxam, LGC-32523, LGC-32533 and LGC-32799 is 0.010 µg/g (ppm) and the limit of detection (LOD) is µg/g (0.005) ppm.

3.5.3 Validation Sample Set

The validation set consisted of the following samples:

Seven instrument calibration standards (0.0070 to 0.320 µg/g)
One reagent blank
Two unfortified control samples
Five samples fortified with ethaboxam, LGC-32523, LGC-32533 and LGC-32799 at 0.010 µg/g (ppm, 1xLOQ)
Five samples fortified with ethaboxam, LGC-32523, LGC-32533 and LGC-32799 at 0.100 µg/g (ppm, 10xLOQ)

3.5.4 Preparation of 1000 µg/mL (PPM) Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 Standard Solution

Section 4.7.1 provides an example calculation describing the preparation of the 1000- µg/mL stock standard solution. Certificates of analysis are presented in Appendix 1.

An aliquot of approximately 0.010g of analyte was weighed out into an amber glass vial. The appropriate amount of methanol was added to the vial to yield a 1000 µg/mL standard solution. All concentrations of the ethaboxam, LGC-32523, LGC-32533 and LGC-32799 standard solutions were stored refrigerated at 2 to 6ºC.

3.5.5 Preparation of Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 Fortification and Calibration Standard Solutions

A 10 µg/mL standard solution was prepared by adding 1.0 mL of the 1000 µg/mL standard to a 100-mL volumetric flask and bringing the solution up to the 100-mL final volume with methanol. From this 10 µg/mL standard, a 1.0 µg/mL standard solution was prepared by combining 10 mL of the 10 µg/mL standard into a 100-mL volumetric flask and bringing the solution up to the 100-mL final volume with methanol. From this 1.0 µg/mL standard, a 0.10 µg/mL standard solution was prepared by combining 0.10 mL of the 1.0 µg/mL standard with 900 µL of methanol. From this 0.10 µg/mL standard, a 0.010 µg/mL standard solution was prepared by combining 0.10 mL of the 0.10 µg/mL standard 0.90 mL of methanol.

3.5.6 Preparation of Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 Instrument Calibration Working Standard Solutions

Seven levels of instrument calibration working standards were prepared (0.7x, 1x, 2x, 5x, 10x, 20x and 32x LOQ) and named with respect to the concentration in the fortified samples (see the table below and the example calculations presented in Section 4.7.3). The standards described in the table below were brought up to a final 1.0-mL volume with HPLC water.

Ethaboxam, LGC-32523, LGC-32533 and LGC-32799 Instrument Calibration Working Standard Solutions

Concentration Relative to the Sample and In-solution Concentration	Concentration Stock Solution (ng/µL)	Volume of Stock Solution (µL)	Final Volume (mL)
0.7xLOQ = 0.04375 µg/L	0.010	17.5	4.0
1xLOQ = 0.0625 µg/L	0.010	25	4.0
2xLOQ = 0.1250 µg/L	0.010	50	4.0
5xLOQ = 0.3125 µg/L	0.100	12.5	4.0
10xLOQ = 0.625 µg/L	0.100	25	4.0
20xLOQ = 1.250 µg/L	0.100	50	4.0
32xLOQ = 2.000 µg/L	0.100	80	4.0

3.5.7 Preparation of Samples

The control soil sample used in this ILV was provided by Valent. Ethaboxam residues were determined in soil samples by extracting the sample with acetone/water (3:1, v/v) and then centrifuging the extracts. The sample volume was adjusted with methanol and then diluted with HPLC-grade water and filtered.

3.5.8 Preparation of Fortification Samples

A 50-µL aliquot of the 1.0 ng/µL (ppm) standard solution was added to each 5 g sample replicate for a 1x LOQ fortification. A 50-µL aliquot of the 10 ng/µL (ppm) standard solution was added to each 5 g replicate for a 10x LOQ fortification. Section 4.7.2 presents the calculations used to prepare the fortified samples.

3.5.9 Analysis Procedure

The analysis procedure was performed as described in method RM-49S-1 with a minor change. The sample extract final volume was changed from 45 mL to 50 mL. An excerpt of the report containing the method is incorporated into the Study Protocol which is presented in Appendix 2.

3.5.10 LC-MS/MS Operating Parameters

Mass Spectrometer Conditions for Positive Ion Analysis

Interface	:	TurboIonSpray
Polarity	:	Positive
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	600ºC
Ionspray voltage	:	5000
Collision gas setting (CAD)	:	Nitrogen set at 6 (arbitrary units)
Gas 1 (GS1)	:	Air set at 70 (arbitrary units)
Gas 2 (GS2)	:	Air set at 70 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**Ethaboxam 1 Primary Transition**	**Ethaboxam 2 Confirmatory Transition 1**	**d5-Ethaboxam Primary Transition**	**LGC-32523 Primary Transition**	**LGC-32533 Primary Transition**
Q1 *m/z*	:	321.0	321.0	326.1	200.0	310.0
Q3 *m/z*	:	200.1	182.9	205.0	129.1	201.0
Dwell time	:	400 ms	400 ms	200 ms	400 ms	200 ms
Resolution Q1	:	Unit	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	82 V	82 V	60 V	69 V	72.9 V
Entrance potential (EP)	:	11.5 V	11.5 V	10.0 V	11.0 V	8.3 V
Collision energy (CE)	:	35.5 V	32.9 V	40 V	34.2 V	25.4 V
Collision cell exit potential (CXP)	:	12 V	12 V	12 V	7.8 V	12.0 V

Column: Two Zorbax Eclipse XDB-C18, 4.6 x 50 mm, 5-micron analytical columns. One column was used for the positive ion analytes and the second was used for the negative ion analyte.

Guard Column : Two Phenomenex, P/N AJO-4287, Security Guard Cartridge, C-18, 4 x 3.0 mm ID. One guard column was used for the positive ion analytes and the second was used for the negative ion analyte.

Injection volume: 30 µL (40 uL into a 30-µL injection loop)
Initial Flow Rate: mL/min: 0.400
Mobile Phase A: 0.05% formic acid in HPLC water
Mobile Phase B: 0.05% formic acid in HPLC methanol

Gradient:	Module:	Events:	Parameter:
Time: 0.0	pumps	Pump B Conc	20%
Time: 0.30	pumps	Pump B Conc	20%
Time: 1.00	controller	Event 3	(Effluent to MS)
Time: 1.00	pumps	Pump B Conc	35%
Time: 2.00	pumps	Pump B Conc	50%
Time: 3.00	pumps	Pump B Conc	50%
Time: 6.80	pumps	Pump B Conc	85%
Time: 7.00	pumps	Total Flow	0.400
Time: 7.50	controller	Event 1	(Effluent to Waste)
Time: 7.50	pumps	Total Flow	0.700
Time: 10.80	pumps	Total Flow	0.700

Time: 10.80	pumps	Pump B Conc	90%
Time: 11.30	pumps	Total Flow	0.400
Gradient:	Module:	Events:	Parameter:
Time: 11.30	pumps	Pump B Conc	20%
Time: 15.00	controller	Stop	

Expected Retention time: LGC-32523, 2.7 min., LGC-32533, 6.0 min.,
d5-Ethaboxam, 6.4 min., Ethaboxam, 6.5 min.

Analyte:	Ethaboxam	d5-Ethaboxam	LGC-32523	LGC-32533
Quantifier m/z:	321.0/200.1	326.1/205.0	200.0/129.1	310.0/201.0
Qualifier m/z:	321.0/182.9	na	na	na

Copies of example chromatograms are included in the Figures Section and the operating parameters for the LC-MS/MS are included in Appendix 3.

Mass Spectrometer Conditions for Negative Ion Analysis

Interface	:	TurboIonSpray
Polarity	:	Negative
Curtain gas (CUR)	:	Nitrogen set at 30 (arbitrary units)
Temperature (TEM)	:	600ºC
Ionspray voltage	:	-4500
Collision gas setting (CAD)	:	Nitrogen set at 8 (arbitrary units)
Gas 1 (GS1)	:	Air set at 70 (arbitrary units)
Gas 2 (GS2)	:	Air set at 70 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**LGC-32799 Primary Transition**
Q1 *m/z*	:	320.0
Q3 *m/z*	:	107.9
Dwell time	:	500 ms
Resolution Q1	:	Unit
Resolution Q3	:	Unit
Declustering potential (DP)	:	-74 V
Entrance potential (EP)	:	-8.3 V
Collision energy (CE)	:	-39.5 V
Collision cell exit potential (CXP)	:	-4.75 V

Column: Two Zorbax Eclipse XDB-C18, 4.6 x 50 mm, 5-micron analytical columns. One column was used for the positive ion analytes and the second was used for the negative ion analyte.

Guard: Phenomenex, P/N AJO-4287, Security Guard Cartridge, C-18, 4 x 3.0 mm ID. One guard column was used for the positive ion analytes and the second was used for the negative ion analyte.

Injection volume: 30 µL (40 uL into a 30 uL injection loop)

Flow Rate: mL/min: 0.400
Mobile Phase A: 5 mM NH_4OAc
Mobile Phase B: HPLC methanol

Gradient:	Module:	Events:	Parameter:
Time: 0.00	pumps	Pump B Conc	50%
Time: 1.00	pumps	Pump B Conc	50%
Time: 3.00	pumps	Pump B Conc	90%
Time: 3.00	controller	Event 3	(Effluent to MS)
Time: 5.50	controller	Event 1	(Effluent to Waste)
Time: 7.00	pumps	Pump B Conc	90%
Time: 7.50	pumps	Pump B Conc	50%
Time: 10.00	controller	Stop	

Expected Retention time: LGC-32799, 4.1 min

Analyte: LGC-32799

Quantifier m/z: 320.0/107.9
Qualifier m/z: na

Copies of example chromatograms are included in the Figures Section and the operating parameters for the LC-MS/MS are included in Appendix 3.

Calibration Procedures

Instrument calibration working standard solutions were prepared as described in Section 3.5.6. Seven instrument calibration working standards were positioned within the analytical batch sequence. The standard concentrations were prepared at 0.7x, 1x, 2x, 5x, 10x, 20x and 32x LOQ (0.007, 0.010, 0.020, 0.050, 0.10, 0.20 and 0.32 µg/g, ppm, respectively). The Analyst® Software (version 1.6) generated a linear (with 1/x weighting) calibration curve and the associated correlation coefficient (r) for each analyte, except ethaboxam, by plotting the analyte peak area count versus analyte concentration. For ethaboxam, The Analyst® Software (version 1.6) generated a linear (with 1/x weighting) calibration curve and the associated correlation coefficient (r), by plotting the analyte peak area count/internal standard peak area versus analyte concentration. The correlation coefficient (r) was required to be greater than or equal to 0.995. The equation generated by Analyst® Software (version 1.6) was verified using Microsoft® Excel (2003).

3.5.11 Data Acquisition and Reporting

The analysis of samples by LC-MS/MS generated electronic data via the Analyst® Software (version 1.6) interface. The hardware, security, and report configurations were set through the software modules. These modules also enabled instrument tuning, provided a mechanism for setting the acquisition methods and batches, processed the data, and quantified the data.

The Analyst Software generated raw data files from which the data were tabulated, and the chromatograms and the standard curves were generated. The data and the resulting descriptive statistics are summarized in Tables 1 through 5 (Tables Section). Representative chromatograms are presented in the Figures Section.

3.5.12 Qualifier Ions

Data were collected for the qualifier ion of ethaboxam 321.0/182.9 m/z.