2.0 INTRODUCTION

The purpose of this study was to validate Methods LKF 114 and LKF 115, "Validation of Methodology for the Post-registration Monitoring of Residues of LGC-30473 in Soil" [1] and "Validation of Methodology for the Post Registration Monitoring of Residues of LGC-30473 in Drinking, Ground and Surface Water" [2], respectively.

This study was designed to fulfill the requirements of the U.S. EPA's Ecological Effects Test Guideline: Data Reporting for Environmental Chemistry Methods, OPPTS 850.7100 [3]. This study was conducted in compliance with U.S. EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160 [4].

3.0 MATERIALS AND METHODS

3.1 Test Substance

Chemical name:	*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide
CAS number:	162650-77-3
Lot number:	AS 2293a
Stated purity:	100%
Expiration date:	08 February 2012
Storage conditions:	Frozen at ≤-20°C

3.2 Test Matrices

Untreated soil used for the validation method was obtained from a terrestrial field dissipation study conducted by Valent U.S.A. Corporation near Madera, California (a loamy sand) and supplied by the Sponsor. Soil was stored at ambient temperature in Storage Cabinet 1 until needed for analysis. Untreated water used for the validation method was collected from the tap in the CPS laboratory on an as-needed basis.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Methods LKF 114 and LKF 115 (Methods Sections). Identical or equivalent apparatus and materials were used.

3.3.1 Equipment and Apparatus

-80°C Freezer (SANYO)
Analytical Balance (Mettler Toledo)
Beckman Coulter Allegra X-22R Centrifuge
Column—Phenomenex Luna® 1.50 × 2.0 mm, 5 µm
Electronic Micro Pipettor 1000 µL (Biohit)

Heraeus® Megafuge® 11R Centrifuge (Thermo)
HPLC System (Agilent 1200®)
Manual Micro Pipettor 10 µL (BrandTech®)
Manual Micro Pipettor 100 µL (BrandTech®)
Manual Micro Pipettor 10,000 µL (VWR)
Manual Micro Pipettor 5000 µL (VWR)
Nitrogen-Evaporator (Organomation Associates, Inc.)
Refrigerator/Freezer (Nor-Lake Scientific)
Tandem Mass Spectrometer, MS/MS (Applied Biosystems® API 4000™)
Top-loading Balance (Mettler Toledo)
Wrist-Action® Shaker (Burrell Model 75)

3.3.2 Reagents

Acetic Acid (J.T. Baker®)
Acetonitrile (EMD®)
Ammonium Acetate (EMD®)
Dichloromethane (EMD®)
Ethyl Acetate (EMD®)
Hexane (EMD®)
HPLC Water (EMD®)
Methanol (Pharmco-AAPER)

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILVs, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined.

One request for clarification was made by the Study Director to the Sponsor regarding water and hexane aliquot measurements. It was determined that 20 mL of water and 10 mL of hexane was correct. Additional clarification was requested regarding reconstitution before quantification by LC-MS/MS. It was determined that a small reconstitution volume followed by proper dilution for analysis would be appropriate. Another request for clarification was made by the Study Director to the Sponsor regarding the fortification level in water. It was determined that fortification levels at 1 µg/L and 10 µg/L were correct. Another communication with the Sponsor addressed sample preparation methods and analytical sequences. A final communication with the Sponsor addressed an error investigation regarding the water analysis. The error investigation revealed the standard curve was made incorrectly. It was determined that re-preparing the standard curve from the initial stock standard solution and reanalyzing the original fortification extractions would be acceptable. It was agreed that, as the error was not related to the method, the reanalysis was still considered trial number one.

3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

3.4.2.1 Sample Validation Sets

The analytical sets consisted of 12 samples: two control samples, five control samples fortified at the LOQ of 1 ng/mL for water or 50 ng/g for soil, and five control samples fortified at 10× LOQ (10 ng/mL for water or 500 ng/g for soil).

All validation samples were assigned a unique identification number during preparation and analysis.

Data are summarized in Table 1 (water) and Table 2 (soil). Residue data sheets are included in Appendix 1.

3.4.2.2 Fortification

Control samples were fortified with 10.0-µg/mL fortification standard solutions.

3.4.2.3 Extraction and Workup

The following extraction steps were followed for each sample.

Water

1. Measure 100 mL tap water into a 250-mL separating funnel.
2. Fortify with appropriate stock solutions, if required.
3. Add 50 mL dichloromethane.
4. Shake vigorously by hand for 1 minute, and let the funnel sit for approximately 3 to 5 minutes to separate the phases.
5. Collect the dichloromethane layer (bottom) into a 250-mL beaker.
6. Repeat steps 3 through 5 once, and combine the dichloromethane extract in the same beaker.
7. Add portions of the extract to a 50-mL test tube for evaporation in the nitrogen evaporator at approximately 40°C. Continue adding portions until all of the extract is evaporated.
8. Rinse the beaker once with 10 mL dichloromethane after transferring the last portion, and transfer to the same test tube for evaporation to dryness.
9. Reconstitute the sample in 20 mL of a solution of 50/50 methanol/water. Further appropriate dilution in solution of 50/50 methanol/water may be performed before quantification by LC-MS/MS.

Soil

1. Weigh 20 g soil into a 250-mL polyethylene or polypropylene bottle.
2. Fortify with appropriate stock solutions, if required.
3. Add 80 mL extraction solvent (70/30 acetonitrile (ACN)/water).

4. Shake for approximately 30 minutes using a wrist-action shaker.
5. Centrifuge the sample at 4000 RPM for 5 minutes.
6. Filter into a 200-mL volumetric flask through a funnel plugged with glass wool.
7. Repeat steps 3 through 6, and combine the extractions.
8. Adjust the final volume to 200 mL in the flask with extraction solvent, stopper, and mix thoroughly.
9. Using a pipette, transfer 10 mL of extract to a 50-mL tube and add 20 mL water.
10. Add 10 mL hexane.
11. Shake vigorously for 1 minute.
12. Centrifuge at 4000 RPM for 5 minutes to separate phases.
13. Discard the hexane layer (top).
14. Repeat steps 10 through 13 once.
15. Add 10 mL ethyl acetate.
16. Shake vigorously for 1 minute.
17. Centrifuge at 4000 RPM for 5 minutes to separate phases.
18. Collect the ethyl acetate layer (top) into a 50-mL test tube.
19. Repeat steps 15 through 18, and combine the extractions (total volume of approximately 20 mL).
20. Evaporate to dryness at approximately 40°C using a nitrogen evaporator.
21. Reconstitute the sample in 5 mL of a solution of 50/50 methanol/water. Further appropriate dilution in solution of 50/50 methanol/water may be performed before quantification by LC-MS/MS.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described by Methods LKF 114 [1] and LKF 115 [2].

3.4.4 Fortification and Standard Solution Preparation

A stock solution of the analytical standard, ethaboxam (V-10208, LGC-30473; 100% purity) was prepared by measuring 11.50 mg of the analytical standard into a 10-mL volumetric flask and bringing to volume with ACN. An intermediate stock containing 10 µg/mL analyte was prepared by measuring 87 µL of the primary stock into a 10-mL volumetric flask and bringing to volume with ACN. A fortification standard was also prepared at a concentration of 0.1000 µg/mL by measuring 100 µL of the intermediate stock into a 10-mL volumetric flask and bringing to volume with ACN.

Standard calibration solutions were prepared at nine concentrations ranging from 1 to 50 ng/mL in 50/50 methanol/water. All solutions were stored in a refrigerator when not in use.

3.5 LC/MS/MS Instrumentation

Instrumentation
HPLC System (Agilent 1200®)
Tandem Mass Spectrometry, MS/MS (Applied Biosystems® API 4000™)
Software: Applied Bio-Systems, Analyst 1.5.1

3.6 Data Acquisition and Reporting

Peak integration and quantification were performed by Analyst software version 1.5.1. Analyte quantification was achieved by external calibration. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for each analyte. A best-fit, no weighting, linear (through zero) regression equation was derived and used to calculate the concentration of the analyte in each sample. The correlation coefficients for the calibration curves for each analytical set were greater than 0.99. Recovery results were computed for each sample. The equations used for quantification are presented in Appendix 2. A statistical treatment of the data includes the calculation of averages, standard deviations, and RSDs. Mean percent recoveries, standard deviations, and RSDs were calculated using Microsoft® Office Excel 2003. Results were rounded off for reporting purposes but not during calculations.

Table 3 HPLC System Operating Parameters

HPLC System:	Agilent Model 1200®
Software:	Applied Bio-Systems, Analyst 1.5.1
Analytical Column:	Phenomenex Luna® 1.50 × 2.0 mm, 5 µm
Column Temperature:	Room Temperature
Injection Volume:	20 µL
Run Time:	25.0 minutes
Mobile Phase:	(A): 80/20 (v/v) HPLC-grade water/ACN, 0.01 M ammonium acetate, 0.1% acetic acid (B): 20/80 (v/v) HPLC-grade water/ACN, 0.01 M ammonium acetate, 0.1% acetic acid

Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.00	60.0	40.0	200
6.00	0.0	100.0	200
12.00	0.0	100.0	200
13.00	60.0	40.0	200
25.00	60.0	40.0	200

Table 4 MS/MS Operating Parameters

Tandem Mass Spectrometry System, Applied Biosystems® API 4000™
Software: Applied Bio-Systems, Analyst 1.5.1

The following parameters were used for operation of the mass spectrometer:

Parameter	Setting
Ion Source:	TurboSpray Ionization (ESI)
Scan Type:	MRM
Polarity:	Positive
Curtain Gas (CUR):	40.00
Temperature (TEM):	400.00
Ion Spray Voltage (IS):	5500.00
Collision Gas (CAD):	10.00
Ion Source Gas 1 (GS1):	40.00
Ion Source Gas 2 (GS2):	40.00
Interface Heater (ihe):	Off
Declustering Potential (DP):	90.00
Entrance Potential (EP):	11.00
Transitions Monitored:	(Q1) 321.000→(Q3) 200.000 m/z quantitative
(Q1) 321.000→(Q3) 200.000 m/z	
Collision Energy (CE):	35.00
Collision Cell Exit Potential(CXP):	14.00

APPENDIX 2 Calculations

Peak integration and quantification were performed by Analyst software version 1.5.1. Analyte quantification was achieved by external calibration. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analyte of interest. A best-fit, no weighting, linear (through zero) regression equation was derived and used to calculate the concentration of the analyte in each sample. The recoveries of the analyte from fortified samples were calculated as follows:

Linear regression formula from calibration curve $y = mx + b$

$$\text{ng/mL analyte} = \frac{y - b}{m}$$

where y = Sample peak area
b = Calibration intercept (in this case, b = 0)
m = Slope

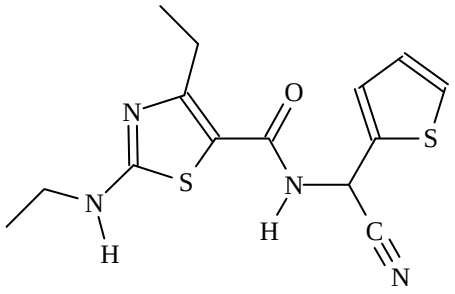
Note: The equations below are written for water samples. For soil samples, "ng/mL found" would change to "ng/g found" and "mL sample" would change to "g sample".

$$\text{ng/mL found} = \text{ng/mL of analyte} \times \frac{\text{mL solvent}}{\text{mL sample}} \times \frac{\text{mL final volume}}{\text{mL aliquot}} \times \text{extra dilution factor}$$

$$\text{Percent Recovery} = \frac{\text{concentration of analyte (ng/mL) - concentration of analyte control (ng/mL)}}{\text{analyte fortification level (ng/mL)}} \times 100$$

Performing Laboratory Test Substance Reference Number

Standard Name	:	Ethaboxam (V-10208, LGC-30473)
Lot		AS 2293a
Chemical Name	:	*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide
CAS Number	:	162650-77-3
Molecular Formula	:	$C_{14}H_{16}N_4OS_2$
Molecular Weight	:	320



Other

Upon completion of the final study, a copy of the protocol and the final report will be archived at CPS. The original protocol, final report, raw data, correspondence, and other documentation will be transferred to Valent U.S.A. Corporation, 1600 Riviera Avenue, Suite 200, Walnut Creek, California, 94596.