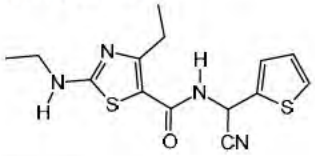
MATERIALS AND METHODS

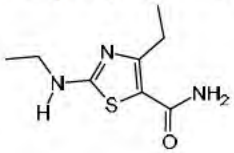
1.1 TEST SUBSTANCE/REFERENCE STANDARDS

The reference standards that were used for the validation are described as follows:

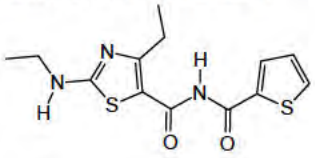
Active Ingredient: Ethaboxam, LGC-30473
Chemical Name [IUPAC]: N-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide
CAS#: 162650-77-3
Active Ingredient Structure:



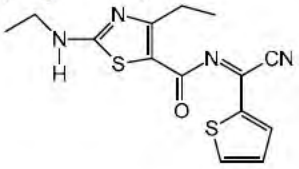
Designation: LGC-32523
Chemical Name [IUPAC]: 4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide
Active Ingredient Structure:



Designation: LGC-32533
Chemical Name (IUPAC): 4-ethyl-2-(ethylamino)-N-(thiophen-2-ylcarbonyl)-1,3-thiazole-5-carboxamide
Active Ingredient Structure:



Active Ingredient: LGC-32799
Chemical Name [IUPAC]: N-[(Z)-cyano(thiophen-2-yl)methylidene]-4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide
Active Ingredient Structure:



The reference standard certificates of analysis are included in APPENDIX 3 and summarized in the following table:

Reference Standard	CAS Number	Lot Number	% Purity	Expiration Date
Ethaboxam	162650-77-3	AS 2293a	100	February 3, 2016
LGC-32523	NA	AS 2313b	97.5	July 23, 2016
LGC-32533	NA	AS 2315b	99.9	July 24, 2016
LGC-32799	NA	AS 2410a	97.8	October 6, 2015

1.2 TEST SYSTEM

The test system used for the validation was a control soil sample from Valent study V-13-38327 collected at Madera County, California. The sample was stored in a freezer (ca. -20°C) when not in use.

1.3 EQUIPMENT AND REAGENTS

The equipment and reagents used for the method validation were as outlined in the method presented in APPENDIX 2. Specific equipment and materials used in this validation are listed below.

1.3.1 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading
Centrifuge
Centrifuge tubes, polypropylene, 50 mL graduated with caps (BD Falcon #2098)
Column: Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902)
Freezer, -20°C capable
Glass bottles, 4 oz amber with Teflon-coated caps (VWR Cat. No. 36319-435)
Glass vials (approximately 22 mL)
Guard Column (optional): C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series)
Mass Spectrometer (Applied Biosystems API 4000)
Pipette(s), automatic - capable of accurately dispensing volumes of 0.050 to 10 mL
Pipette(s), volumetric, delivery head or bottle-top (20 mL)
Reciprocating mechanical shaker, (Erbach)
Refrigerator
Syringe, polypropylene, 3 mL
Syringe filter, PTFE 0.2 micron

1.3.2 REAGENTS

Acetone
Ammonium acetate
Formic acid
Methanol
Water (HPLC-grade)

EXPERIMENTAL PROCEDURES

1.4 STANDARD SOLUTIONS PREPARATION

Stock standard solutions were prepared from the neat reference standards for use in the preparation of fortification solutions and instrument calibration solutions. All standard solutions were prepared as per the method. The stock standards were stored refrigerated (ca. 4°C) when not in use.

1.5 SAMPLE PREPARATION

All samples were prepared as per the method.

The analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified at the LOQ (0.0100 mg/kg), and five untreated controls fortified at 10×LOQ (0.100 mg/kg).

1.6 SAMPLE ANALYSIS

All samples were analyzed as per the method except one column was used for both positive and negative ion mode analyses. The specific instrumentation and settings for method validation are listed below. Conditions were optimized following the validation and the optimized conditions are presented in the analytical method.

1.6.1 INSTRUMENTATION

Ethaboxam, LGC-32523, LGC-32533 HPLC Conditions (Positive Ion Mode):

Guard Column:	C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289)
Column:	Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902)
Column temperature:	25 ºC
Sample temperature:	ambient
Mobile Phases:	HPLC-grade Water, 0.05% Formic Acid (v/v) Methanol, 0.05% Formic Acid (v/v)
Injection Volume:	25 µL
Divert:	0 – 1.3 minute (waste) 1.3 minutes (MS)
Split:	none

Gradient Program:

Time (minutes)	% HPLC-grade Water (0.05% Formic Acid)	% Methanol (0.05% Formic Acid)	Flow Rate: (µL/minute)
0.0	65	35	400
1.0	65	35	400
5.0	10	90	400
7.5	10	90	400
8.0	65	35	400
11.5	65	35	400

Retention times (approximate):

LGC-32523	2.0 minutes
LGC-32533	5.9 minutes
Ethaboxam	6.8 minutes

MS-MS Conditions:

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	15 psi(N_2)
Gas sources: GS1 =	25 psi(N_2), GS2: 25 psi(N_2)
Ion spray voltage (IS):	2000 V
Temperature (TEM):	450 °C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
LGC-32523	200.0	129.0	500	75	10	40	12
LGC-32533	310.3	201.0	500	65	10	30	10
Ethaboxam	321.1	200.0	400	60	10	40	12
Ethaboxam 2	321.1	183.0	300	60	10	40	12
d5-Ethaboxam	326.1	205.0	150	60	10	40	12

LGC-32799 HPLC Conditions (Negative Ion Mode):

Guard Column:	C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289)
Column:	Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902)
Column temperature:	25 ºC
Sample temperature:	ambient
Mobile Phases:	5mM Ammonium acetate in HPLC-grade Water Methanol
Injection Volume:	25 µL
Divert:	0 – 4 minutes (waste) 4 – 8 minutes (MS) 8 – 10 minutes (waste)
Split:	none

Gradient Program:

Time (minutes)	5mM Ammonium acetate in HPLC-grade Water	% Methanol (0.05% Formic Acid)	Flow Rate: (µL/minute)
0.0	50	50	400
1.0	50	50	400
3.0	10	90	400
7.0	10	90	400
7.5	50	50	400
10.0	50	50	400

Retention time (approximate):
LGC-32799 6.4 minutes

MS-MS Conditions:

Scan Type: MRM
Mode: Negative
Ion source: Turbo V^{TM}
Probe Type: Electrospray
Collision gas (CAD): 8 psi(N_2)
Curtain gas (CUR): 15 psi(N_2)
Gas sources: GS1 = 25 psi(N_2), GS2: 25 psi(N_2)
Ion spray voltage (IS): -4000 V
Temperature (TEM): 450 °C
Interface heater (IH): On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
LGC-32799	321.8	107.8	500	-75	-10	-35	-5

1.7 CALCULATIONS

Analyst Chromatography Software (Analyst ver. 1.6.1; Applied Biosystems, Foster City, CA) was used to acquire and integrate the detector responses for each injection. The peak area $Area_{Analyte}$ and $Area_{ISTD}$ were entered into an EXCEL® spreadsheet to calculate the data.

To calculate the curve for instrument calibration, the area ratio (ethaboxam/d5-ethaboxam(ISTD)), the peak area (LGC-32523, LGC-32533 and LGC-32799), and the nominal concentration of each of the calibration standards were input into an Excel spreadsheet. A weighted second order polynomial standard curve ($Y=aX^2+bX+c$) was generated for each analyte with each set of analyses, and the coefficients (*a, b, c*) of the curve were determined. The curve was used to calibrate the instrument, determine the acceptability of the standard injections and to calculate the sample analyte concentrations. The curve was generated by plotting the standard detector response (Ethaboxam = $Area_{STD}/Area_{ISTD}$; LGC-32523, LGC-32533 and LGC-32799 = $Area_{STD}$) versus the nominal standard concentration and was weighted relative to the largest standard concentration.

Five different standard concentrations were injected within each analytical set. The concentrations (µg/L) of ethaboxam, LGC-32523, LGC-32533 and LGC-32799 detected in sample extracts were interpolated from the respective standard calibration curves. Analyte concentrations for the standards were calculated by the Excel spreadsheet using the equation:

$$C_{standard}\ (\mu g/L) = a \times [\text{Detector response}]^2 + b \times [\text{Detector response}] + c$$

Where: $C_{standard}$: concentration of analyte in the standard solution, (µg/L);
a, b, c: curve constants;
Detector response: ethaboxam = $Area_{STD}/Area_{ISTD}$; all other analytes = $Area_{STD}$

For samples, the residue concentration in the sample (ppm, mg/kg, µg/g) was calculated using the equation:

$$\text{Sample Concentration, } (\mu\text{g/g}) = \frac{[aX^2 + bX + c\,] \text{ x C x D x E}}{\text{F x G}} \times \frac{1\text{ L}}{1000\text{ mL}}$$

where: X = Detector response (Area Ratio or Peak Area)
a = constant (for x^2 term in polynomial fit)
b = constant (for x term in polynomial fit)
c = constant (for polynomial fit)
C = Final volume (4 mL)
D = Dilution factor, used if the sample extract is diluted prior to analysis
E = Extract volume prior to aliquot taken (45 mL)
F = Aliquot volume taken (0.25 mL)
G = Sample weight (5.0 g)

Percent recoveries for the fortified samples were corrected for the average detector response observed in the associated control samples. The recovery was corrected using either the average area ratio (ethaboxam/d5-ethaboxam (ISTD)) or the average peak area (all other analytes) observed in the control samples. The average detector response was subtracted from the detector response in the fortified sample prior to calculating a corrected concentration. There were no untreated control detector responses above the respective lowest calibration standard detector response in this study.

The corrected fortified sample recovery was calculated as follows:

$$\text{Fortified Recovery} = \frac{\text{Corrected Fortified Sample Concentration}}{\text{Theoretical Fortified Sample Concentration}} \times 100\%$$

An example calculation for an ethaboxam fortified untreated control sample (sample Ft 1), in set V-13-38327-1a, fortified at 0.01 mg/kg, is as follows:

Corrected detector response: area ratio Ft 1 – average area ratio in controls 8U1 utc1 and 8U1 utc2 = 0.213-0.013 = 0.200

$$\text{Ft 1 Concentration, } (\mu\text{g/g}) = \frac{[aX^2 + bX + c\,] \text{ x C x D x E}}{\text{F x G}} \times \frac{1\text{ L}}{1000\text{ mL}}$$

where: X = corrected detector response = 0.200
a = constant (for x^2 term in polynomial fit) = -1.69E-04
b = constant (for x term in polynomial fit) =3.20E-01
c = constant (for polynomial fit) = 5.78E-04
C = Final volume = 4 mL
D = Dilution factor = 1
E = Extract volume prior to aliquot taken = 45 mL
F = Aliquot volume taken = 0.25 mL
G = Sample weight 5.0 g

$$Conc\ Ft1 = \frac{[-1.69E-04(0.200)^2 + 3.20E-01(0.200) + 5.78E-04]\ x\ 4\ x\ 1\ x\ 45}{0.25\ x\ 5.0} \times \frac{1\ L}{1000\ mL}$$

$$= \frac{11.6228}{1.25}\ X\ \frac{1.000}{1000}$$

Concentration of Ft 1(µg/g) = 0.0093

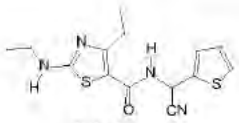
1. INTRODUCTION

This method describes the determination of ethaboxam (*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide), LGC-32523 (4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide) LGC-32533 (4-ethyl-2-(ethylamino)-*N*-(thiophen-2-ylcarbonyl)-1,3-thiazole-5-carboxamide) and LGC-32799 (*N*-[(*Z*)-cyano(thiophen-2-yl)methylidene]-4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide) in soil. The method involves sample extraction with acetone/water (3:1, v/v) and centrifugation to separate the solids. Sample volumes are adjusted with methanol and an aliquot is diluted with HPLC-grade water. A portion is filtered using syringe filtration and transferred into an autosampler vial. Samples are analyzed using high-performance liquid chromatography with tandem mass spectrometry LC/MS-MS (with turbo-ion spray ionization in positive and negative ion modes).

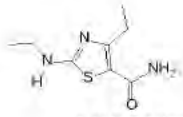
2. MATERIALS

2.1 Analytical Reference Standards

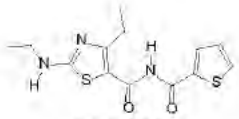
The following analytical reference standards are used:



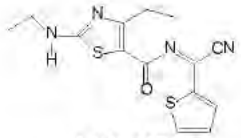
Ethaboxam



LGC-32523



LGC-32533

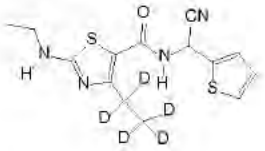


LGC-32799

Designation:	Ethaboxam (aka V-10208; Valent USA Corporation)
IUPAC name:	*N*-(cyano-2-thienylmethyl)-4-ethyl-2-(ethylamino)-5-thiazolecarboxamide
Designation:	LGC-32523
IUPAC name:	4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide
Designation:	LGC-32533
IUPAC name:	4-ethyl-2-(ethylamino)-*N*-(thiophen-2-ylcarbonyl)-1,3-thiazole-5-carboxamide

Designation: LGC-32799
IUPAC name *N*-[(*Z*)-cyano(thiophen-2-yl)methylidene]-4-ethyl-2-(ethylamino)-1,3-thiazole-5-carboxamide

Internal Standard



d5-Ethaboxam

2.1.1 Analytical Reference Standard Preparation

Other quantities may be prepared as long as proportions are maintained.

Individual Analyte Stock Solutions, 1.0 mg/mL

Weigh 0.100 grams of each analyte (ethaboxam, LGC-32523, LGC-32533 and LGC-32799) into separate 100-mL volumetric flasks. [To ensure a 1.0 mg/mL concentration, correct the amount of standard weighed (or the volume of solvent used) for the purity of the standard.] Dilute each flask to volume with acetone or methanol. The standards may be transferred into glass containers with Teflon-lined caps for storage. Store in a refrigerator at ≤ 10 °C.

Mixed Analyte Standard Solution, 10 mg/L

Transfer 1.00 mL of each of the 1.0 mg/mL stock solutions (the stock solutions of ethaboxam, LGC-32523, LGC-32533 and LGC-32799) into a 100-mL volumetric flask. Dilute to volume with methanol. The standard may be transferred into a glass container with a Teflon-lined cap for storage. Store in a refrigerator at ≤ 10 °C.

Mixed Analyte Standard Solution, 1 mg/L

Transfer 10.0 mL of the 10 mg/L mixed analyte standard solution of ethaboxam, LGC-32523, LGC-32533 and LGC-32799 into a 100-mL volumetric flask. Dilute to volume with methanol. The standard may be transferred into a glass container with a Teflon-lined cap for storage. Store in a refrigerator at ≤ 10 °C. This standard solution must be validated according to Valent SOP VR-003 "Analytical Standard Solutions".

d_5-Ethaboxam internal standard stock solution, 20 mg/L

Weigh 0.4 milligrams (0.0004g) of d_5-ethaboxam into suitable container. [Asume the purity of the standard is 100%.] Pipet 20 mL of acetone or methanol into the flask. The

standards may be transferred into glass containers with Teflon-lined caps for storage. Store in a refrigerator at ≤ 10 °C.

d_5-Ethaboxam working internal standard solution, 0.01 mg/L

Transfer 100 µL of the 20 mg/L d_5-ethaboxam internal standard solution into a 200-mL volumetric flask. Dilute to volume with methanol. The standard may be transferred into a glass container with a Teflon-lined cap for storage. Store in a refrigerator at ≤ 10 °C.

Working Calibration Standard Solutions

The working calibration standard solutions may be prepared in the following manner (by serial dilution, starting from the 1 mg/L mixed analyte standard). Other concentrations may be used as necessary.

Freshly Prepared Working Calibration Standards in HPLC Water

Starting Intermediate Standard Solution, Concentration (mg/L)	Volume of Intermediate Standard Solution Used (mL)	Working Calibration Standard Solution Total Volume (mL)	Working Calibration Standard Solution Concentration (µg/L)
1	0.08	20	4
0.004	10	20	2
0.002	2.5	10	0.5
0.0005	5	10	0.25
0.00025	3	10	0.075

The working calibration standard solutions are prepared in HPLC-grade water using pipettes and suitable glass containers. Mix each standard well. Transfer a portion into an autosampler vial. The standards are prepared fresh daily and do not require validation according to Valent SOP VR-003 "Analytical Standard Solutions".
Into a separate suitable container, pipet 0.250 mL of the 0.01 mg/L working internal standard solution, 1.75 mL of HPLC water, and 2.0 mL of the working calibration standard solution. Mix well. Transfer each final mixture into two separate autosampler vials, if needed (One set of vials for analysis of LGC-32799 and another set for an analysis of the other three analytes).

Fortifications

Typically, the 1 mg/L mixed analyte standard solution is used for preparing fortified control samples.

For example: Prepare a 0.01 ppm fortified control sample by adding 0.050 mL of the 1.0 mg/L intermediate standard into 5.0 g of crop sample.

2.2. Reagents

Reagents must be of adequate quality (pesticide or HPLC-grade) to perform the analytical procedures.

Acetone
Ammonium acetate
Formic acid
Methanol
Water (HPLC-grade)

2.2.1 Reagent Solution Preparation

Reagent solutions may be prepared in the following manner. Other volumes may be used, provided that the correct proportions are maintained.

Acetone/Water (3:1, v/v).

Add 3 parts acetone with 1 part HPLC-grade water. For example, add 750 mL of acetone and 250 mL of water sequentially into a reagent bottle. Mix well and store at room temperature.

Ethaboxam, LGC-32523, LGC-32533 (Positive Ion Mode) Mobile Phases:

HPLC Eluent – HPLC-grade Water, 0.05% Formic Acid (v/v).

Add 0.500 mL of formic acid to 1000 mL of HPLC-grade water in a reagent bottle. Mix well and store at room temperature.

HPLC Eluent - Methanol, 0.05% Formic Acid (v/v).

Add 0.500 mL of formic acid to 1000 mL of methanol in a reagent bottle. Mix well and store at room temperature.

LGC-32799 (Negative Ion Mode) Mobile Phases:

HPLC Eluent – 5mM Ammonium acetate in HPLC-grade Water,

Add 0.385 g of ammonium acetate to a 1 L volumetric flask. Swirl and sonicate to dissolve solids. Dilute to 1L with HPLC-grade water. Mix well, transfer to a reagent bottle and store at room temperature.

HPLC Eluent - Methanol

Add 1000 mL of methanol into a reagent bottle. Store at room temperature.

3 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading

Centrifuge
Centrifuge tubes, polypropylene, 50 mL graduated with caps (BD Falcon #2098 or equivalent)
Columns (2): Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902) or equivalent
Freezer, -20°C capable
Glass bottles, 4 oz amber with Teflon-coated caps (VWR Cat. No. 36319-435 or equivalent)
Glass vials (approximately 22 mL or equivalent)
Guard Column (optional): C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289, or equivalent)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), automatic - capable of accurately dispensing volumes of 0.050 to 10 mL
Pipette(s), volumetric, delivery head or bottle-top (20 mL)
Reciprocating mechanical shaker, (Erbach or equivalent)
Refrigerator
Syringe, polypropylene, 3 mL (or equivalent)
Syringe filter, PTFE 0.2 micron

4 INSTRUMENTATION

High Performance Liquid Chromatograph with Mass Spectrometry (LC/MS-MS) – Agilent Technologies 1200 series HPLC with tandem Applied Biosystems API 4000 mass selective detector, with turbo ion spray ionization in positive and negative ion modes. HPLC conditions shown below are the optimized conditions, which are slightly different than method validation conditions, and are suggested for this analysis. The conditions may be modified as needed to optimize the chromatography, to resolve matrix interferences, or to utilize other types of LC/MS-MS instruments. The LC/MS-MS parameters that are used must be documented with each chromatographic set. **It is highly recommended to use separate columns for positive and negative mode analyses. Also the system should be flushed without a column, prior to equilibration with the column, when switching from negative to positive mode analyses.**

Ethaboxam, LGC-32523, LGC-32533 HPLC Conditions (Positive Ion Mode):

Guard Column (optional):	C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289, or equivalent)
Column 1:	Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902)
Column temperature:	25 °C
Sample temperature:	ambient
Mobile Phases:	HPLC-grade Water, 0.05% Formic Acid (v/v) Methanol, 0.05% Formic Acid (v/v)
Injection Volume:	25 µL
Divert:	0 – 1 minute (waste) 1 – 7.5 minutes (MS) 7.5 minutes (waste)
Split:	none

Gradient Program.

Time (minutes)	% HPLC-grade Water (0.05% Formic Acid)	% Methanol (0.05% Formic Acid)	Flow Rate: (µL/minute)
0.0	65	35	400
1.0	65	35	400
2.0	50	50	400
3.0	50	50	700
6.8	10	90	700
10.8	10	90	700
11.3	65	35	700
11.8	65	35	400
15.0	65	35	400

The retention times listed below are based on the suggested parameters above and differ slightly from the example chromatograms.

Retention times:

LGC-32523	2.0 minutes
LGC-32533	6.0 minutes
Ethaboxam	6.4 minutes

MS-MS Conditions:

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	15 psi(N_2)
Gas sources: GS1 =	25 psi(N_2), GS2: 25 psi(N_2)
Ion spray voltage (IS):	2000 V
Temperature (TEM):	450 °C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
LGC-32523	200.0	129.0	500	75	10	40	12
LGC-32533	310.3	201.0	500	65	10	30	10
Ethaboxam	321.1	200.0	400	60	10	40	12
Ethaboxam 2	321.1	183.0	300	60	10	40	12
d5-Ethaboxam	326.1	205.0	150	60	10	40	12

LGC-32799 HPLC Conditions (Negative Ion Mode):

Guard Column (optional):	C8, 2.0 X 4.0 mm (Phenomenex Part # AJO-4289, or equivalent)
Column 2:	Eclipse XDB-C18 5µm, 4.6 x 50 mm (Agilent Part # 946975-902)
Column temperature:	25 °C
Sample temperature:	ambient
Mobile Phases:	5mM Ammonium acetate in HPLC-grade Water Methanol
Injection Volume:	25 µL
Divert:	0 – 4 minutes (waste) 4 – 8 minutes (MS) 8 – 10 minutes (waste)
Split:	none

Gradient Program:

Time (minutes)	5mM Ammonium acetate in HPLC-grade Water	% Methanol	Flow Rate: (µL/minute)
0.0	50	50	400
1.0	50	50	400
3.0	10	90	400
7.0	10	90	400
7.5	50	50	400
10.0	50	50	400

Retention time (approximate):

LGC-32799	6.4 minutes

MS-MS Conditions:

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	15 psi(N_2)
Gas sources: GS1 =	25 psi(N_2), GS2: 25 psi(N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	450 °C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
LGC-32799	321.8	107.8	500	-75	-10	-35	-5

5 ANALYTICAL PROCEDURES

1. Sample Setup

Allow the homogenized bulk samples to completely thaw in the bulk sample bag. Mix the thawed bulk sample well making sure not to spill the contents. Weigh 5.0 g ± 0.1 g of sample into a 50-mL polypropylene centrifuge tube by sampling from at least 3 different places within the bulk sample bag. At this point, a control sample to be used for method recoveries may be fortified using the appropriate standard solution. Note: Samples may be weighed into 50-mL polypropylene centrifuge tubes and stored frozen (ca.-20°C) prior to fortification and analysis.

2. Extraction

Add ca. 20 mL of acetone/water (3:1, v/v) to each 50-mL polypropylene centrifuge tube, cap tightly, and place on a reciprocal shaker for thirty (30) minutes. Centrifuge for five (5) minutes at a speed that can pelletize the solids (for example, 4000 revolutions per minute (rpm) or at 3313 relative centrifugal force (rcf)). Decant the supernatant into a second graduated 50-mL polypropylene centrifuge tube. Add an additional 20 mL of acetone/water (3:1, v/v) to the first 50-mL polypropylene tube, if needed, manually shake vigorously to disintegrate the pellet and repeat the process. Combine extracts in the second 50-mL tube. Discard the first tube. Note: Other suitable graduated containers may be used.

3. Dilution

Adjust the final volume in each graduated tube to 45 mL using methanol. Cap and mix well. Into a separate suitable container, pipet 0.25 ml of 0.01 mg/L internal standard (IS), 3.5 mL of HPLC-grade water, and 0.25 mL of the sample extract. Mix well. Note: Different volumes may be used as long as the same proportions are maintained. For high concentration samples, an additional dilution may be required.

4. Sample Filtration and Injection

Using a disposable polypropylene syringe and a 0.2 micron PTFE syringe filter, filter and transfer at least 1 mL of the sample into two separate autosampler vials, if samples will be analyzed in both positive and negative ion modes, for analysis by LC-MS/MS. Note: Samples may be transferred into a single vial if vial caps are capable of sustaining multiple injections. For some soil types, more than one 0.2 micron syringe filter may be required.

A set of 25 previously weighed samples will require approximately 3 hours preparing for LC-MS/MS analysis.

6 LC/MS-MS ANALYSIS

Instrument calibration is performed using either a linear fit with a non-zero intercept or a 2^{nd}-order polynomial fit (weighted relative to 1/concentration). The calibration is performed with calibration standards that are distributed (interspersed with the sample extracts) within each analytical sequence.

For a 2nd-order polynomial calibration, analyze a minimum of five calibration standard concentrations within the analytical sequence (for linear n = 4). A typical set of standards should include concentrations of 0.125, 0.250, 1, 2 and the required 0.0375 µg/L standard (with an injection volume of 25 µL).

The coefficient of determination (r^2) is calculated from these calibration standards. This value must be greater than 0.99 for the instrument response to be considered acceptable over the range of concentrations. In addition, the concentration calculated from the detector response of each of the standards, using the linear or the 2nd-order polynomial fit, must be within 15% of the theoretical standard concentration, unless approved by the supervising chemist or Study Director.

Additional continuing calibration standards (typically a mid-range calibration standard at 0.250 µg/L for linear or 2nd-order polynomial calibrations) may also be analyzed as part of the analytical sequence. Typically, the sequence is constructed with the following order: a continuing calibration standard, 2 to 6 prepared samples, a continuing calibration standard or a calibration standard, 2 to 6 prepared samples, and a continuing calibration standard. The sequence must begin and end with a continuing calibration standard. A 0.250 µg/L calibration standard is analyzed in the middle of the sequence and also evaluated as a continuing calibration standard. This ensures a minimum of three continuing calibration standard responses for evaluation. The coefficient of variation (CV) of the continuing calibration standard responses must be 15% or less for the analytical set to be acceptable, unless approved by the supervising chemist or Study Director.

If the detector response (Ethaboxam = $Area_{STD}/Area_{ISTD}$; LGC-32523, LGC-32533 and LGC-32799 = $Area_{STD}$) observed for a sample is greater than the detector response of the highest calibration standard, the sample extract must be diluted and the diluted extract analyzed. The sample extract must be diluted such that the peaks obtained are within the calibrated response range of the LC/MS-MS.

7 CALCULATIONS

To calculate the curve for instrument calibration, the area ratio (ethaboxam/d_5-ethaboxam), the peak area (LGC-32523, LGC-32533 and LGC-32799), and the nominal concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to either a linear or a 2nd-order polynomial regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte area ratio or peak area. Replicate entries are included in the data set prior to performing the regression in Excel (to provide weighting relative to 1/concentration).

For example:

Calibration Standard	Relative Weighting Calcn (High Std Conc / Std Conc)	Number of Entries in Data Set
2 µg/L	2 / 2	1
1 µg/L	2 / 1	2
0.25 µg/L	2 / 0.250	8
0.125 µg/L	2 / 0.125	16
0.0375 µg/L	2 / 0.0375	53

The curve is used to calibrate the instrument, determine the acceptability of the standard injections and to calculate the sample analyte concentrations. The curve is generated by plotting the standard detector response (Ethaboxam = $Area_{STD}/Area_{ISTD}$; LGC-32523, LGC-32533 and LGC-32799 = $Area_{STD}$) versus the nominal standard concentration and is weighted relative to the largest standard concentration.

For a 2nd-order polynomial calibration, analyte concentrations for the standards are calculated by the Excel spreadsheet using the equation:

$$C_{standard}\ (\mu g/L) = a \times [\text{Detector response}]^2 + b \times [\text{Detector response}] + c$$

Where:

$C_{standard}$:	concentration of analyte in the standard solution, (µg/L)
a, b, c:	curve constants
Detector response:	ethaboxam = $Area_{STD}/Area_{ISTD}$; all other analytes = $Area_{STD}$

For a linear calibration, the concentration in the sample is calculated as follows:

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX + b] \times C \times D \times E}{F \times G} \times \frac{1\,L}{1000\,mL}$$

where:

X = Sample response (Area ratio or Peak Area)
a = slope
b = intercept
C = Final volume (4 mL)
D = Dilution factor, used if the sample extract is diluted prior to analysis
E = Extract volume prior to aliquot taken (45 mL)
F = Aliquot volume taken (0.25 mL)
G = Sample weight (5.0 g)

For a 2nd-order polynomial calibration, the concentration in the sample is calculated as follows:

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX^2 + bX + c] \times C \times D \times E}{F \times G} \times \frac{1\,L}{1000\,mL}$$

where: X = Sample response (Area Ratio or Peak Area)
a = constant (for x^2 term in polynomial fit)
b = constant (for x term in polynomial fit)
c = constant (for polynomial fit)
C = Final volume (4 mL)
D = Dilution factor, used if the sample extract is diluted prior to analysis
E = Extract volume prior to aliquot taken (45 mL)
F = Aliquot volume taken (0.25 mL)
G = Sample weight (5.0 g)

For calculation of analyte recovery in a fortified sample, the recovery is corrected by using either the area ratio (ethaboxam/d_5-ethaboxam), peak area or the concentration observed in the control sample. If the area ratio or peak area in the control sample is equal to or greater than the lowest calibration standard, then the concentration observed in the control sample is subtracted from the concentration observed in the fortified sample to provide a corrected concentration. Otherwise, the area ratio or peak area in the control sample is subtracted from the area ratio or peak area in the fortified sample prior to calculating a corrected concentration. This corrected concentration is then used to calculate percent recovery:

$$\text{Percent Recovery} = \frac{\text{Corrected Concentration Observed in Fortified Sample}}{\text{Theoretical Concentration in Fortified Sample}} \text{ x } 100\%$$

For evaluation of the continuing calibration standards (with a minimum of three interspersed within the analytical sequence), the average detector response (or calculated concentration) and the standard deviation for these standards is calculated. The coefficient of variation (CV) is then calculated to evaluate the reproducibility of the instrument over the analytical sequence:

$$\text{Coefficient of Variation, \%} = \frac{\text{Standard Deviation, peak unit or area ratio}}{\text{Average Response, peak unit or area ratio}} \text{ x } 100\%$$

8 LIMIT OF DETECTION

The limit of detection (LOD) of this method is 0.005 ppm. The detection limit is based on a 5.0 g sample weight, a 0.25-mL aliquot (of the 45-mL extract volume), a 4-mL final volume, and a 0.0375 µg/L calibration standard (as the lowest concentration in the set of calibration standards):

$$\text{Limit of Detection} = \frac{4\text{ mL Final Vol. x } 0.0375\ \mu\text{g/L Stnd}}{5.0\text{ g Sample Wt.}} \text{ x } \frac{1\text{ L}}{1000\text{ mL}} \text{ x } \frac{45\text{ mL Extract Vol.}}{0.25\text{ mL Aliquot}} = 0.005\text{ ppm}$$

9 CHROMATOGRAMS

Example chromatograms are shown in Figures 1 through 16.