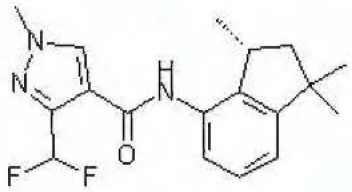
2 MATERIALS AND METHODS

2.1 TEST SUBSTANCE/REFERENCE STANDARDS

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

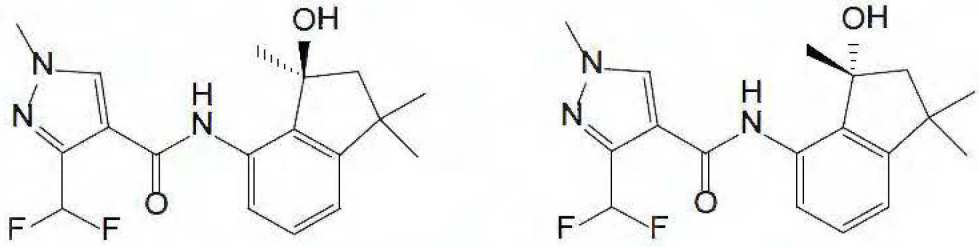
Active Ingredient: S-2399
Chemical Name: 3-(Difluoromethyl)-1-methyl-*N*-[(3'*R*)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1*H*-pyrazole-4-carboxamide

Valent lot #: AS 2375a
Active Ingredient Structure:

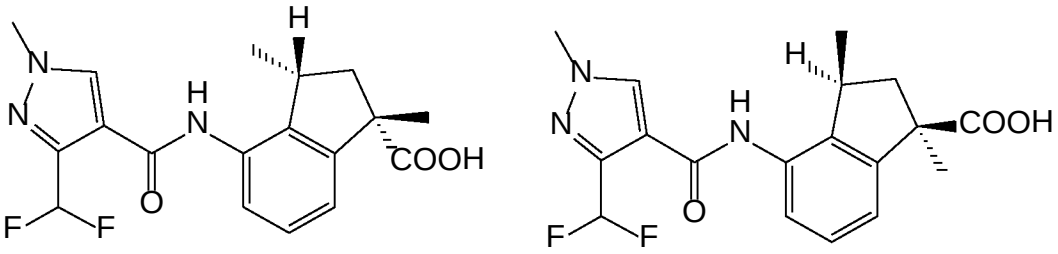


Active Ingredient: 3'-OH-S-2840
Chemical Name : 3-(Difluoromethyl)-*N*-[3'-hydroxy-(3'S)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1-methyl-1*H*-pyrazole-4-carboxamide
3-(Difluoromethyl)-*N*-[3'-hydroxy-(3'R)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1-methyl-1*H*-pyrazole-4-carboxamide

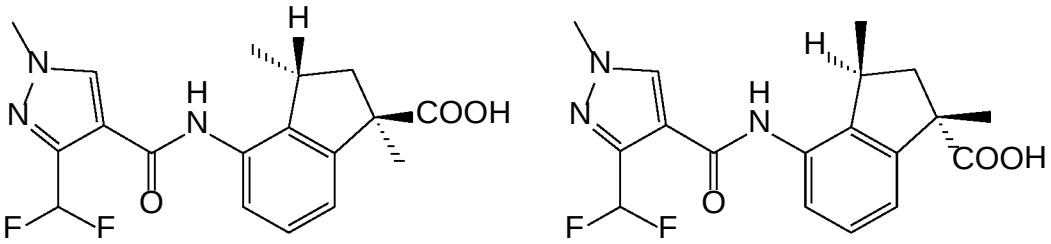
Valent Lot #: AS2379a
Active Ingredient Structure:



Active Ingredient: 1'-COOH-S-2840-A
Chemical Name : 4′-({[3-(Difluoromethyl)-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′H-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′H-indene-1′-carboxylic acid
Valent Lot #: AS2393a
Active Ingredient Structure:



Active Ingredient: 1'-COOH-S-2840-B
Chemical Name : 4´-({[3-(Difluoromethyl)-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)-(1´R,3´R)-1´,3´-dimethyl-2´,3´ dihydro-1´H-indene-1´-carboxylic acid
4´-({[3-(Difluoromethyl)-1-methyl-1H-pyrazol-4-yl]carbonyl}amino)-(1´S,3´S)-1´,3´-dimethyl-2´,3´ dihydro-1´H-indene-1´-carboxylic acid
Valent Lot #: AS2394a
Active Ingredient Structure:



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

Reference Standard	Lot Number	% Purity	Expiration Date
S-2399	AS 2375a	96.0	10/11/2015
3'-OH-S-2840	AS 2379a	99.7	6/2/2017
1'-COOH-S-2840-A	AS 2393a	100	2/12/2016
1'-COOH-S-2840-B	AS 2394a	99.6	2/12/2016

2.2 TEST SYSTEM

The test system used for the validation was a control soil sample from Valent study V-38603-A, 8U-1. The samples were stored in a freezer when not in use.

2.3 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.

2.3.1 EQUIPMENT

Analytical balance (Mettler Toledo)
Top-loading balance (Mettler Toledo)
Centrifuge, Sorvall Evolution RC, (thermo Scientific)
Centrifuge Polypropylene conical tubes, 50-mL (VWR)
Dispensing pipette – capable of dispensing 25 mL
Glass funnels, 70 mm
Glass wool
LC/MS-MS

Agilent 1260 Binary Pump HPLC system with an autosampler coupled to an Applied Biosystems API 4000 mass spectrometer with an electrospray ionization interface.

Walk-in -20°C freezer (Imperial Brown, Inc.)
Refrigerator/freezer (Nor-Lake® Scientific)
Graduated cylinders, various sizes
Pasteur pipets, various sizes
Pipettor, Automatic, 20 µL to 10 mL volumes, (Rainin)
Reciprocating shake, (Eberbach)
Round-bottom boiling flasks, 500 mL
Rotary Evaporators with temperature controlled water baths
Separatory Funnels, 250 mL
Ultrasonic cleaner, 3200 (Branson)
Vials, 20 mL screw-capped vials with caps
Vials, autosampler
Volumetric flasks, various sizes
Volumetric Pipettes, assorted volumes

2.3.2 REAGENTS

Acetone, (EMD)
Ammonium Acetate (Aldrich)
Dichloromethane (EMD)
Hydrochloric Acid (JT Baker)
Methanol (EMD)
Sodium Acetate trihydrate (Aldrich)
Sodium Chloride, (VWR)
Sodium Sulfate (EMD)
Water, HPLC (EMD)

3 EXPERIMENTAL PROCEDURES

3.1 STANDARD SOLUTIONS PREPARATION

Stock standard solutions were prepared from the neat reference standard for use in the preparation of fortification solutions and instrument calibration solutions. Optional internal standards were also prepared and the calibration standards were diluted with the internal standard solutions. All standard solutions were prepared as per the method. As allowed by method the fortifying solutions, calibration standards, and internal standards were prepared in larger volumes. Due to low quantity of the neat substances, the stock standard solutions were weighed into a vial then acetone (or methanol for

internal standards) was added to make a 1.0 mg/mL solution. When preparing a calibration solution, the 1 ug/mL intermediate solution was made by directly adding 100 uL of each of the 1 mg/mL stock solution into a 100 mL volumetric flask and was diluted to volume with the 0.001 ug/mL internal standard solution. These types of variations are allowed by the method. The standard solutions were stored in a refrigerator when not in use.

3.2 SAMPLE PREPARATION

All samples were prepared as per the method presented in APPENDIX 2.

The analytical set for soil consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified at the LOQ (0.01 mg/kg), and five untreated controls fortified at 10×LOQ (0.1 mg/kg).

The first analytical set for soil consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified at the LOQ (0.01 mg/kg), and five untreated controls fortified at 10×LOQ (0.1 mg/kg). One of the 0.01 mg/kg fortification samples for 1'-COOH-S-2840-B was not within the 70-120% recovery range and a second smaller set consisting of one untreated control and three untreated controls fortified at the LOQ (0.01 mg/kg) was analyzed for 1'-COOH-S-2840-B only.

3.3 SAMPLE ANALYSIS

All samples were analyzed as per the method presented in APPENDIX 2. Samples were bracketed by calibration standards. The continuing standards ran just before the first calibration standard with no sample in between them, in the middle, and just after the last calibration standard with no sample between them.

3.3.1 INSTRUMENTATION

LC/MS-MS

Agilent 1260 Binary Pump HPLC system with an autosampler coupled to an Applied Biosystems API 4000 mass spectrometer with an electrospray ionization interface.

Analytical Column

Eclipse XDB-C8, 5µm, 150 mm x 4.6mm, Agilent part # 993967-906

Column Oven Temperature:	40 ± 1°C
Mobile Phase:	A = 5mM ammonium acetate in HPLC water B = 5mM ammonium acetate in methanol
Gradient Program:	T = 0 min, 65% A + 35% B T = 1.0 min, 65% A + 35% B T = 6.0 min, 10% A + 90% B T = 7.0 min, 35% A + 65% B T = 10.0 min, 35% A + 65% B T = 11.0 min, 65% A + 35% B T = 15.0 min, 65% A + 35% B
Flow Rate Program:	700 µL/min

Injection,

Drawing Speed:	200 µL/minute
Injection Volume:	25 µL
Ejecting Speed:	200 µL/minute

Period 1

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V^{TM}
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	10 psi(N_2)
Gas sources: GS1 =	20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	500 °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)
1'-COOH-S-2840-A	362	318	200	-10	-10	-18	-5
1'-COOH-S-2840-A 2	362	131	200	-10	-10	-30	-5
d3-1'-COOH-S-2840-A	365	321	200	-10	-10	-18	-5
1'-COOH-S-2840-B	362	318	200	-10	-10	-18	-5
1'-COOH-S-2840-B 2	362	131	200	-10	-10	-30	-5
d3-1'-COOH-S-2840-B	365	321	200	-10	-10	-18	-5

Period 2

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V^{TM}
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	10 psi(N_2)
Gas sources: GS1 =	20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	500 °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)
3'-OH-S-2840	348	175	400	-10	-10	-23	-5
3'-OH-S-2840 2	348	130	400	-65	-10	-35	-10
d3-3'-OH-S-2840	351	178	400	-10	-10	-23	-5

Period 3

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V^{TM}
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	10 psi(N_2)
Gas sources: GS1 =	20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS):	4000 V
Temperature (TEM):	500 °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)
S-2399	334	258	400	55	10	27	19
S-2399 2	334	238	400	55	10	45	19
d3-S-2399	337	261	400	55	10	27	19

4.1 CALCULATIONS

All samples were calculated as per the method.

4.2 DEVIATIONS

1. Changes from sample handling data form that was attached to the protocol that occurred were:

5 mL 0.2M sodium carbonate added to graduated cylinder before addition of acid extraction had been replaced with 2 mL of 0.5 M sodium acetate added to graduated cylinder after addition of acid extraction.

Internal standards have been added as an option.

Addition of “immediate” timing for procedures for the acidic extraction steps.

These changes made the method perform better.

2. In the laboratory, the calibration standards were not validated per SOP:VR-003-09. For calibration standards only WS (no MS standards) were made. These standards were validated by plotting each analyte (S-2399, 3’-OH-S-2840,1’-COOH-S-2840-A, and 1’-COOH-S-2840-B)against their peak area ratios to a weighed polynominal curve. The back-calculated calibration standard concentrations using a non-linear calibration curve were within 15% of prepared concentration. This proves that preparation of the calibration standards is accurate and further validation of them is redundant. This deviation did not impact the study.

VALENT U.S.A. CORPORATION
Valent Technical Center
Dublin, California

Determination of Residues of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Soil
Method: **RM-50S**

Date: November 2, 2015

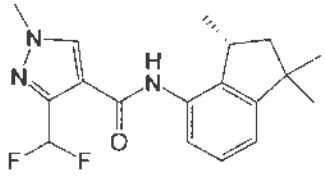
1. INTRODUCTION

This method determines residues of S-2399 and it's metabolites 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in soil. The metabolites that are designated S-2840 are racemic mixtures of enantiomers. For the carboxylic acid metabolites, the A and B designations of the acids are based on their isomeric similarities. The reference standard A contains 2 isomers, 1'S-3'R and 1'R-3'S, which are mirror images of each other, or enantiomers. The standard B also contains 2 isomers, 1'R-3'R and 1'S-3'S, which are also enantiomers. However, the members of A and B are not enantiomers, they are diastereomers, and because of that, the NMR spectra of A and B are not identical. This method is a modification of Sumitomo Chemical Company's Report ER-MT-1422.

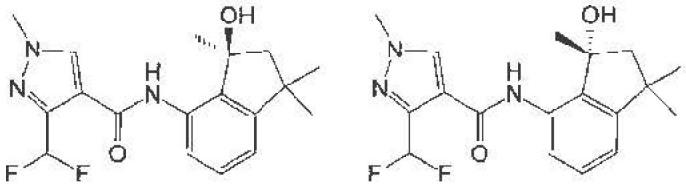
Briefly, the residues are extracted from soil using two extractions of acetone/water (4/1, v/v) followed by an extraction with acetone/0.5M HCl (4/1, v/v). The residues are partitioned into dichloromethane. The dichloromethane from this step is evaporated, and the residues are quantitated by LC/MS/MS. The method also includes conditions for the optional use of internal standards.

2. MATERIALS

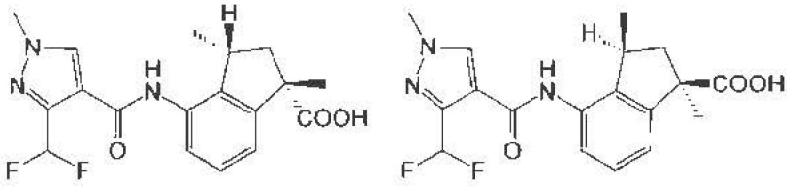
2.1. ANALYTICAL REFERENCE STANDARDS



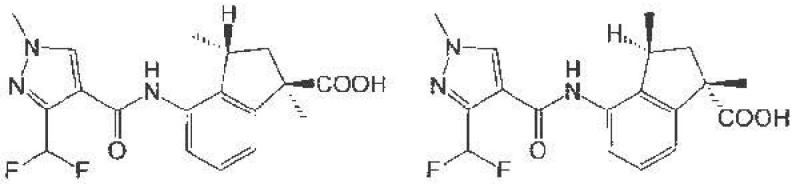
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3′*R*)-1′,1′,3′-trimethyl-2′,3-dihydro-1′*H*-inden-4′-yl]-1*H*-pyrazole-4-carboxamide



3'-OH-S-2840 (MW = 349.4)
3-(Difluoromethyl)-*N*-[3'-hydroxy-(3'R)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1-methyl-1*H*-pyrazole-4-carboxamide
3-(Difluoromethyl)-*N*-[3'-hydroxy-(3'S)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1-methyl-1*H*-pyrazole-4-carboxamide

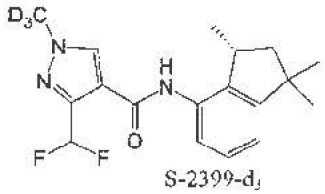


1'-COOH-S-2840-A (MW = 363.4)
4'-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1'R,3'S)-1',3'-dimethyl-2',3' dihydro-1'*H*-indene-1'-carboxylic acid
4'-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1'S,3'R)-1',3'-dimethyl-2',3' dihydro-1'*H*-indene-1'-carboxylic acid

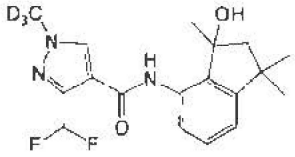


1'-COOH-S-2840-B (MW = 363.4)
4'-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1'R,3'R)-1',3'-dimethyl-2',3' dihydro-1'*H*-indene-1'-carboxylic acid
4'-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1'S,3'S)-1',3'-dimethyl-2',3' dihydro-1'*H*-indene-1'-carboxylic acid

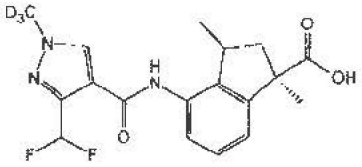
2.2. OPTIONAL INTERNAL STANDARDS



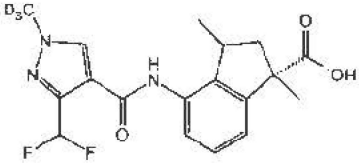
S-2399-d_3



3'-OH-S-2840-d_3



1'-COOH-S-2840-A-d_3



1'-COOH-S-2840-B-d_3

2.3. ANALYTICAL REFERENCE STANDARD PREPARATION

Other quantities may be prepared and other containers and measuring devices (*e.g.*, vials and pipets) may be used as long as proportions are maintained and documented.

Stock Solutions, 1 mg/mL:

For each analyte (S-2399, 3'-OH-S-2840,1'-COOH-S-2840-A, and 1'-COOH-S-2840-B), accurately weigh 10 mg (correct the amount for chemical purity) and transfer to a 10 mL volumetric flask. Dilute with acetone to volume or adjust final volume to ensure a 1.0 mg/mL solution-weight. . If less than 10 mg is available then dispense the known amount into a vial and pipette the appropriate amount of acetone in the vial to ensure a 1.0 mg/mL solution. Store the stock solutions in a refrigerator or freezer when not in use.

Fortification Solution, 10 µg/mL:

Transfer a 1.0 mL aliquot of each of the 1 mg/mL stock solutions to a 100 mL volumetric flask, and dilute to volume with acetone. Store this solution in a refrigerator or freezer when not in use.

Fortification Solution, 1 µg/mL:

Transfer a 10 mL aliquot of the 10 µg/mL fortification solution to a 100 mL volumetric flask, and dilute to volume with acetone. Store this solution in a refrigerator or freezer when not in use.

Calibration Standard Solutions:

10 µg/L: Transfer a 1 mL aliquot of the 1 µg/mL fortification solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

5 µg/L: Transfer a 50 mL aliquot of the 10 µg/L analytical standard solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

2.5 µg/L: Transfer a 50 mL aliquot of the 5 µg/L analytical standard solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

1 µg/L: Transfer a 40 mL aliquot of the 2.5 µg/L analytical standard solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

0.5 µg/L: Transfer a 50 mL aliquot of the 1 µg/L analytical standard solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

0.25 µg/L: Transfer a 50 mL aliquot of the 0.5 µg/L analytical standard solution to a 100 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v).

Note: If optional internal standards are used these above calibration standards would have volumes diluted with Internal Standard Final Volume Solution, 1 µg/L [instead of methanol/HPLC water (1/1, v/v)].

Store these calibration standard solutions in a refrigerator or freezer when not in use. Additional dilutions and/or alternate concentrations may be prepared to generate appropriate standards. The volumes shown above are examples for preparing the standards; other volumes (aliquots and final volumes) may be used.

2.4. OPTIONAL INTERNAL STANDARD PREPARATION

Internal Stock Solutions, 1 mg/mL:

For each analyte (S-2399-d_3, 3'-OH-S-2840-d_3, 1'-COOH-S-2840-A-d_3, and 1'-COOH-S-2840-B-d_3), accurately weigh 10 mg or what is available if less than 10 mg, and transfer to a 10 mL volumetric flask or a vial if less than 10 mg is available. Dilute with methanol to volume if in volumetric flask or pipette appropriate amount of methanol if in vial to ensure a 1.0 mg/mL solution. Store the stock solutions in a refrigerator or freezer when not in use.

Intermediate Internal Standard Solution, 1 µg/mL:

Transfer a 100 µL aliquot of each of the 1 mg/mL stock solutions to a 100 mL volumetric flask, and dilute to volume with methanol. Store this solution in a refrigerator or freezer when not in use.

Internal Standard Final Volume Solution, 1 µg/L:

Transfer a 1.0 mL aliquot of the 1 µg/mL Intermediate Internal Stock Solution to a 1000 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v). Store this solution in a refrigerator or freezer when not in use.

2.5. REAGENTS

Acetone, pesticide quality or equivalent

Ammonium Acetate, reagent grade or equivalent

Dichloromethane, pesticide quality or equivalent

Hydrochloric acid, 12N, pesticide quality or equivalent

Methanol, pesticide quality or equivalent

Sodium acetate trihydrate, reagent grade or equivalent

Sodium Chloride, reagent grade or eqnivalent

Sodium Sulfate, reagent grade or equivalent

Water, HPLC grade

Water, deionized

2.6. REAGENT SOLUTION PREPARATION

Reagent solutions may be prepared in the following manner. Other volumes may be used, provided that the correct proportions are maintained. All prepared solutions should be well mixed and stored at room temperature.

Acetone/ HPLC Water (4:1, v/v)
Add 4 parts acetone with 1 part HPLC water. For example, add 800 mL of acetone and 200 mL of HPLC water sequentially into a reagent bottle.

Acetone/0.5 M HCL (4:1, v/v)
Add 4 parts acetone with 1 part 0.5M HCl. For example, add 800 mL of acetone and 200 mL of 0.5M HCl sequentially into a reagent bottle.

5mM Ammonium Acetate in Methanol
Add 0.385g ammonium acetate into 1 L of methanol.

5mM Ammonium Acetate in HPLC Water
Add 0.385g ammonium acetate into 1 L of HPLC water.

0.5M HCl Solution
Add 41.7mL of 12N HCL into one liter of HPLC water.

0.5 M Sodium Acetate Solution
Add 68 g sodium acetate trihydrate into one liter of HPLC water.

Sodium Chloride:water: 5% (w/v)
Add 50 grams of sodium chloride to 1 L of deionized water and shake until dissolved.

2.7. EQUIPMENT

Balances, Analytical and Top Loading

Bulk Sample Homogenizers:
Robot Coupe® Food Chopper R25T or equivalent

Centrifuge tubes, 50 mL polypropylene

Centrifuge, Sorvall Centrifuge or equivalent

Funnels, 70 mm

Glass wool

Graduated cylinders, (**pre-rinsed with methanol**), 100 mL with stoppers for sample extracts and other various sizes for making solutions

Pasteur pipets, various sizes for transfers

Pipettor, Automatic, capable of accurately dispensing 20 to 250 µL volumes. Rainin or equivalent

Reciprocating shaker Eberbach or equivalent

Round-bottom boiling flasks (**pre-rinsed with methanol**), 250 or 500 ml.

Rotary Evaporators with temperature controlled water baths

Separatory Funnels, (**pre-rinsed with methanol**), 250 mL

Ultrasonic cleaner, Branson 3200 or equivalent

Vials, (**pre-rinsed with methanol**), 20 mL screw-capped vials with caps

Vials, autosampler (with caps)

Volumetric flasks, (**pre-rinsed with methanol**)25, 50, 100 or 1000 mL for preparing analytical standards

Volumetric Pipettes, assorted volumes including 1, 2, 5 and 10 mL

Note: Equivalent equipment may be substituted for the above items. ***Pre-rinsing of all glassware is essential to remove any remaining residues of the analytes after routine glassware washing.***

2.8. INSTRUMENTATION

LC/MS-MS
Hewlett Packard 1260 Binary Pump HPLC system with an autosampler and an Applied Biosystems API 4000 mass spectrometer with an electrospray ionization interface (or an equivalent system).

Analytical Column
Eclipse XDB-C8, 5µm, 150 mm x 4.6mm, Agilent part # 993967-906

3. ANALYTICAL PROCEDURE

Other quantities, equipment, containers, and measuring devices (*e.g.*, vials and pipets) may be used as long as proportions are maintained and documented.

A. Sample Preparation

Mix by hand or homogenize the bulk sample in the presence of dry ice to obtain a homogeneous sample. If homogenized, allow the dry ice to sublime from the sample before taking a subsample for analysis.

Weigh 10.0 g (± 0.1 g) of the homogenized sample into a 50 mL polypropylene centrifuge tube. At this point, if required by the testing facility, control samples for method recovery should be fortified with S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B (See Note 1).

Weigh a sample for determining the moisture content of the soil on a dry weight basis and analyze.

B. Sample Extraction

Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes at approximately 2000 rpm or as needed to separate the

solids from the extraction solvent. Decant the sample extract into a stoppered 100 mL graduated cylinder.

Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes and decant the sample extract into the 100 mL graduated cylinder containing the first extract.

Note: It is important to conduct the third extraction in a timely manner as the 3'-OH-S-2840 residues are not stable when remaining in acidic conditions.

Add 25 mL of acetone/0.5 M HCl (4/1, v/v) to the centrifuge tube containing the sample and shake on reciprocating shaker for 30 minutes. **Immediately** centrifuge the sample for approximately 5 minutes and decant the sample extract into the 100 mL graduated cylinder containing the first two extracts. **Immediately** add 2 mL 0.5 M sodium acetate solution to the graduated cylinder and mix. Bring extracts in graduated cylinder up to 100 mL with acetone/water (4/1, v/v) and mix. The sample may have flocking. This extract is stable and may sit a few hours on benchtop before partition or can be stored in refrigerator (proven to be stable for up to at least two weeks).

C. Sodium Chloride Dichloromethane Partition

Note: It is important to remix the contents of the graduated cylinder just prior to taking the aliquot due to flocking. It is also important to shake the separatory funnels quickly for the first partition, (add the samples to no more than two separatory funnels at a time, shake and then add samples to the next two separatory funnels).

Add 25 mL of 5% sodium chloride solution to the 250 mL separatory funnel. Remix contents of graduated cylinder just prior to taking aliquot. Add 2.5 mL of extract from just mixed extracts in graduated cylinder and 50 mL dichloromethane to the separatory funnel. *(Note: Dichloromethane may be added to the separatory first, depending on analyst preference)* **Immediately** shake for 1 minute and allow to separate. Drain the lower dichloromethane layer through funnel containing approximately 50 grams of sodium sulfate (suspended on a plug of glass wool) and collect into a 250 or 500 mL round bottom flask.

Add an additional 50 mL dichloromethane to the separatory funnel. Shake one minute and allow to separate. Drain the lower dichloromethane layer through funnel containing sodium sulfate into the round bottom flask containing the first extract.

Note: Other quantities may be may be used as long as proportions are maintained and documented. Example: 10 mL of 5% sodium chloride solution. 2 x 20 mL dichloromethane, and 1 mL of sample extract.

D. Final Volume

Evaporate to dryness using a rotary-evaporator and water bath set to ≤ 40°C or other evaporator system.

Re-dissolve the extract in 5.0 mL of methanol/water (1/1, v/v) [or Internal Standard Final Volume Solution, 1 µg/L if using optional internal standards], sonicate, then transfer the extract to a screw cap vial for storage. Store at ≤ 0°C until LC/MS/MS analysis. Transfer a portion of final volume extract to an autosampler vial.

Note: If other quantities are used in Step C then re-dissolve in a proportioned amount. From the above example, the final volume would be 2 mL.

E. Sample Analyses

Analytical Sequence: Condition the LC/MS/MS instrument with at least five injections of a sample extract. (This number of conditions may be reduced if the set is analyzed consecutively behind another set) Prepare an analytical sequence that contains at least five calibration standard concentrations to establish the response of the instruments. A typical sequence would include 0.25, 0.5, 1, 2.5, 5 and 10 µg/L calibration standards and a continuing calibration standard, typically one of the mid-level concentration calibration standards (*i.e.*, 2.5 or 1 µg/L calibration standard). The analytical sequence must begin (after the conditioning injections) and end with a continuing calibration standard. The continuing calibration standard must also be injected at least once within the sequence to verify the instrument reproducibility (See Note 2). Samples and calibration standards are interspersed within the sequence, so that a calibration standard is injected after every one to five sample injections (unless <5 samples then there maybe be no sample in between two standards). A typical sequence would be as follows: 3-5 conditioning injections, continuing calibration standard, one to three samples, calibration standard, one to three sample injections, calibration standard, … and ending with the continuing calibration standard. As residues of S-2399 and 3'-OH-S-2840 can accumulate on surfaces within the LC/MS/MS it is suggested to add five methanol injections at the end of each sequence. Any sample having detector response greater than the largest calibration standard response must be appropriately diluted with methanol/water (1/1 v/v) or Internal Standard Final Volume Solution so that the analyte response will be within the calibration standard range. The diluted sample is then analyzed along with the untreated control sample and high fortification sample from the set.

HPLC Conditions:

Column: Eclipse XDB-C8, 5µm, 150 mm x 4.6mm, Agilent part # 993967-906	
Column Oven Temperature:	40 + 1°C
Mobile Phase:	A = 5mM ammonium acetate in HPLC water
	B = 5mM ammonium acetate in methanol

Gradient Program:
T = 0 min, 65% A + 35% B
T = 1.0 min, 65% A + 35% B
T = 6.0 min, 10% A + 90% B
T = 7.0 min, 35% A + 65% B
T = 10.0 min, 35% A + 65% B
T = 11.0 min, 65% A + 35% B
T = 15.0 min, 65% A + 35% B

Flow Rate Program: 700 µL/min

Injection,
Drawing Speed: 200 µL/minute
Injection Volume: 25 µL
Ejecting Speed: 200 µL/minute

Typical MS-MS Parameters:

Period 1

Scan Type: MRM
Mode: Negative
Ion source: Turbo V™
Probe Type: Electrospray
Collision gas (CAD): 8 psi(N_2)
Curtain gas (CUR): 10 psi(N_2)
Gas sources: GS1 = 20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS): -4000 V
Temperature (TEM): 500 °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)
1'-COOH-S-2840-A	362	318, (131)	200	-10	-10	-18, (-30)	-5
1'-COOH-S-2840-A- *d3*	365	321	200	-10	-10	-18	-5
1'-COOH-S-2840-B	362	318, (131)	200	-10	-10	-18, (-30)	-5
1'-COOH-S-2840-B- *d3*	365	321	200	-10	-10	-18	-5

Period 2

Scan Type: MRM
Mode: Negative
Ion source: Turbo V™
Probe Type: Electrospray

Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	10 psi(N_2)
Gas sources: GS1 =	20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	500 °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)
3'-OH-S-2840	348	175, (130)	400	-10, (-65)	-10	-23 (-35)	-5 (-10)
3'-OH-S-2840-*d3*	351	178	400	-10	-10	-23	-5

Period 3

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi(N_2)
Curtain gas (CUR):	10 psi(N_2)
Gas sources: GS1 =	20 psi(N_2), GS2: 20 psi(N_2)
Ion spray voltage (IS):	4000 V
Temperature (TEM):	500 °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)
S-2399	334	238, (258)	400	55	10	45, (27)	19
S-2399-*d3*	337	241, (261)	400	55	10	45, (27)	19

The instrument parameters shown above are given only as a guide. They may be modified as needed to optimize the chromatography, to resolve matrix interferences (if observed), or to utilize other types of LC/MS-MS instruments. An alternative longer LC method to resolve some matrix issues is included in this method as Note 3. Each set of chromatograms must be clearly labeled with the LC/MS-MS parameters used.

F. Residue Calculations

The concentrations of the analytes (S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B) in each sample extract are calculated using a second-order polynomial equation on the basis of peak area or peak area ratio (S-2399/d3-S-2399) if using internal standards. The data are presented graphically as concentration of the calibration standards verses their peak areas using typically, an Excel® spreadsheet to determine the curve parameters and calculate the sample residues, however other programs that generate

curve fit data may also be used. The area responses or peak area ratios are entered as the *x* values and concentrations as the *y* values to calculate a curve expressed as the following equation:

$$Y = Ax^2 + Bx + C$$

The data are weighted relative (*i.e.*, proportional) to the concentration of the highest standard concentration. (the largest calibration standard concentration is divided by a calibration standard concentration to get the number of entries in a data set). If a number of entry value does not result in a whole number then multiply all number of entries in the data set by a number that creates a whole number for all (for example, the 0.5 standard divided by a 0.04 standard would give an entry number of 12.5, and then all number of entries are then multiplied by 2 make the number entries a whole number). An example of a calibration standard set, with the number of data entries, is shown below.

Standard Concentration	Number of Entries in Data Set
10 µg/L	1
5 µg/L	2
2.5 µg/L	4
1 µg/L	10
0.5 µg/L	20
0.25 µg/L	40

For example, a set of calibration standards gives peak areas as follows:

µg/L	Area
10	83,262
5	42,871
2.5	21,801
1	8,470
0.5	4,212
0.25	2,321

The resulting equation from the Excel spreadsheet is as follows:

$$Y = Ax^2 + Bx + C$$

A = 6.690 E-11
B = 1.144 E-04
C = -2.161 E-03

To ensure that the equation is appropriate, the areas or peak ratios of the calibration standards are entered into this equation and the standard concentrations are calculated. For each standard, the calculated concentration must each be within 15% of the actual concentration. In addition, the coefficient of determination (r^2) must be greater than 0.99. For example (from the above data), the 1 µg/L standard has an area of 8,470 and the calculated concentration (using the equation) is

0.971 µg/L. This is acceptable as this is 97% of the known concentration. The criteria listed above for recalculated concentrations and the coefficient of determination must be met for acceptance of the each analytical set, unless approved by the chemist responsible for the analysis. Sample extract concentrations are also calculated using the equation from the calibration standards. For example, a sample extract for S-2399 with a peak area of 4,272 would have a concentration as follows:

$$\mu g/L = Ax^2 + Bx + C$$

µg/L = (6.690 E-11x 4,272 x 4,272) + 1.144 E-04x 4,272) -2.161 E-03= 0.488

Concentrations of each analyte (S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B) in the sample is calculated using the following formula:

$$ppm = \frac{C \ x \text{EV x } FV \ x \ DF}{W x AV}$$

where:

C = concentration of extract (in µg/L, from equation)
EV = total extraction volume (100 mL)
FV = final volume of extract (5 mL)
DF = dilution factor (if any)
W = sample weight analyzed (10 g)
AV = aliquot volume (2.5 mL)

For example, the concentration in the above example sample (with a calculated extract concentration of 0.488 µg/L) would be calculated as follows:

$$ppm = \frac{(0.000488\ \mu g/mL) \text{ x } (100mL) \text{ x } (5mL)}{(10g)x(2.5mL)} = 0.00976$$

The sample extract concentration of each analyte (S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B) in the fortified samples are calculated as above except the peak area or peak area ratio (or average if multiple control samples) of the control sample divided by dilution of fortified sample (if any) is subtracted from the area response (or area ratio) of the fortified sample. For example, the above sample was fortified with 0.01 ppm and with an area response of 4,272 and an average area of 332 would have a concentration as follows:

µg/L = (6.690 E-11x (4,272-332) x (4,272-332)) + (1.144 E-04x (4,272-332)) -2.161 E-03= 0.450

The concentration of each analyte (S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B) in the fortified sample is calculated by the above ppm calculation. For example the corrected fortified sample with 0.450 µg/L would have a concentration as follows:

$$ppm = \frac{(0.000450\ \mu g/mL) \times (100mL) \times (5mL)}{(10g) \times (2.5mL)} = 0.00899$$

The percent recovery is calculated by taking the ppm found in fortified sample and dividing by the amount the sample was fortified with and multiplying by 100. For the fortification sample fortified at 0.01 ppm, the following values were used to calculate the amount of S-2399 recovered in the fortified sample:

$$\text{Percent recovery (\%)} = \frac{0.00899\ \text{ppm}}{0.01\ \text{ppm}} \times 100\% = 89.9\%$$

If the area of the untreated control sample is greater than the area of the lowest standard:

The recoveries for fortified samples are calculated using the formula:

$$\text{Percent recovery (\%)} = \frac{\text{ppm in fortified sample - ppm in control sample}}{\text{fortification level}} \times 100$$

For a fortification sample fortified at 1.0 ppm, the following values were utilized to calculate the amount of each analyte in the sample:

ppm found in fortified sample = 0.870
ppm found in untreated control sample = 0.01096

$$\text{Percent recovery (\%)} = \frac{0.870 - 0.01096}{1.0} \times 100 = 86.0\%$$

Samples are corrected for moisture content on a dry weight basis.

$$\text{\% water content} = \frac{\text{(sample wet weight (g) - sample dry weight (g)} \times 100\%}{\text{sample dry weight (g)}}$$

$$\text{Soil dry weight (g)} = \frac{\text{Sample wet weight(g)} \times 100\%}{(100\% + \text{\%water content})}$$

For example a soil with a wet weight of 10 g and a dry weight of 8.5 then the following values would be obtained:

$$\text{\% water content} = \frac{(10(g) - 8.5(g)) \times 100\%}{8.5(g)} = 17.65\%$$

$$\text{Soil dry weight (g)} = \frac{10(g) \times 100\%}{(100\% + 17.65\%)} = 8.50g$$

The concentration (ppm) found in the soil is then corrected to ppm found in dry soil.

$$\text{ppm dry soil} = \frac{\text{ppm found in wet soil} \times (100\% + \%\ \text{water content})}{100\%}$$

If a wet weight concentration of 0.5 ppm was found in a sample and a water content of 17.65% then the following dry weight concentration would be obtained:

$$\text{ppm dry soil} = \frac{0.5\ \text{ppm} \times (100\% + 17.65\%)}{100\%} = 0.588\ \text{ppm}$$

4. ANALYTICAL LIMITS

A. Limit of Detection

The limit of detection (LOD) of this method is 0.005 mg/kg (ppm). This LOD is calculated by dividing the lowest calibration standard concentration (0.00025 ug/mL) by the effective matrix concentration in the sample extracts. This is based on a 10 g sample, a 2.5 mL aliquot (of the 100mL total volume after extraction), a 5 mL final volume, and a 0.00025 ug/mL standard in the calibration.

$$LOD = \frac{(5\text{mL}) \times (100\text{mL}) \times (0.00025\text{ug/mL})}{(2.5mL) x (10g)} = 0.005\text{ppm}$$

B. Limit of Quantitation

The limit of quantitation (LOQ) of this method is 0.01 ppm, based on the lowest fortification level validated in this method.

5. ANALYSIS TIME

A trained analyst, familiar with this method, can complete the analysis of a set of 12 samples in approximately 8 hours. The results are available within 24 hours of initiating the analysis.

6. NOTES

A. Note 1

The level of fortification is generally at the method LOQ (0.01 ppm) and/or at 10 times the LOQ (0.1 ppm). If residues higher than 10 times LOQ are anticipated, then fortifications should be made at a higher concentration (typically slightly above the highest residues). Method recoveries must be 70% to 120% to be acceptable unless approved by the chemist responsible for the analysis. If the testing facility does not

require concurrent analysis of fortified control samples, or if a UTC sample is not available, this method requirement may be waived.

Note 2

At Valent U.S.A. Corporation, reproducibility of an analytical run is determined by calculating the coefficient of variation (CV) from the calculated concentration obtained from the continuing calibration standards analyzed in the analytical sequence (set). For the analytical set to be acceptable, these CV's must be 15% or less unless approved by the chemist responsible for the analysis.

Note 3

An additional longer LC method has been utilized to resolve some matrix issues and help to control accumulation of residues in the instrument.

Gradient Program:	T = 0 min, 50% A + 50% B
	T = 1.0 min, 50% A + 50% B
	T = 6.0 min, 10% A + 90% B
	T = 16.0 min, 10% A + 90% B
	T = 17.0 min, 50% A + 50% B
	T = 27.0 min, 50% A + 50% B
Flow Rate Program:	400 µL/min