SUMMARY

This analytical method validation report has been amended to incorporate modifications based on the recommendations of the independent laboratory validation study (Reference 1). Method RM-50S-2 is a modified version of Valent analytical method RM-50S-1. In this modified version, notes are provided in section 5, ANALYTICAL PROCEDURES during steps 3 and 4 of the method to facilitate analyte percent recoveries. In step 3, centrifugation is recommended if a precipitate is observed during the pH adjustment with buffer solution. In step 4, additional 5-ml rinses with methanol are encouraged to ensure that a complete transfer of the analytes will occur. The final analyte dilution solvent was also clarified as being methanol/HPLC water (1/1,v/v) when an internal standard solution was not in use. If optional internal standards are used, samples are diluted with the 2 µg/L ISFV (Internal Standard Final Volume) solution [instead of methanol/HPLC water (1/1, v/v)]. Additional minor modifications were included in the method for clarification. All modifications (including method modifications) are documented in the report amendment (APPENDIX 6).

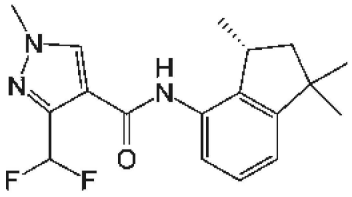
The validation of residue analytical method RM-50S-1, entitled "S-2399: Validation of Valent Method RM-50S-1, "Determination of S-2399, 3'-OH- S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Sediment and Soil", was completed. This method has a limit of detection (LOD) of 0.005 µg/g, and a limit of quantification (LOQ) of 0.010 µg/g, for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in sediment and soil. The protocol for this method validation is included in APPENDIX 1, and the method (including sample calculations and example chromatograms) is presented in APPENDIX 2.

This method was validated by fortification of untreated sediment from study VP-38970, entitled "Aquatic Field Dissipation of S-2399 Following Foliar Application of S-2399 2.84 SC to a Flooded Rice Field in Louisiana", with S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B at nominal concentrations of 0.010 µg/g (LOQ) and 0.100 µg/g (10xLOQ). Analyte recoveries were calculated using internal standards. The recoveries for all analytes are included in the analytical data in APPENDIX 5, and are also presented in Summary Tables I and II.

MATERIALS AND METHODS

1.1 TEST SUBSTANCE/REFERENCE STANDARDS

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



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

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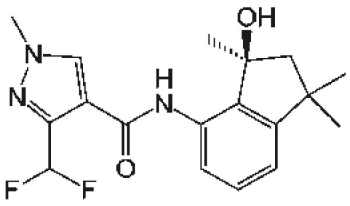

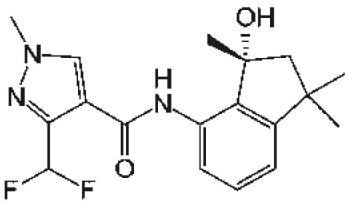

3'-OH-S-2840 (MW = 349.4)

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

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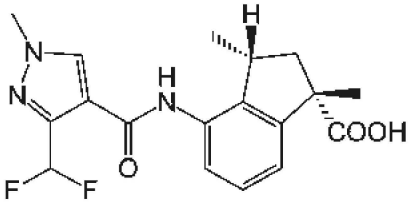
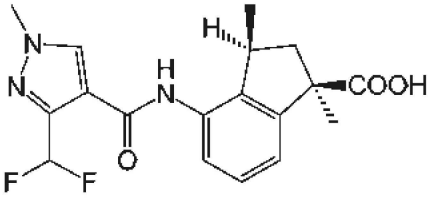
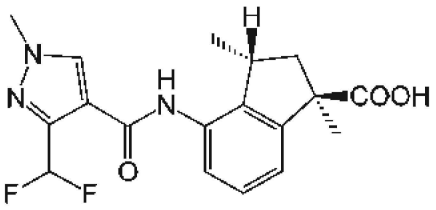
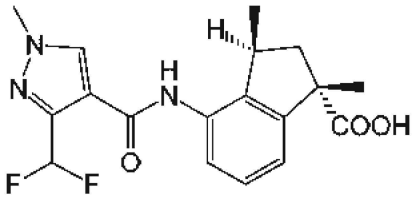




1'-COOH-S-2840-A (MW = 363.4)

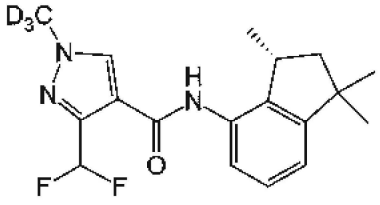
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid

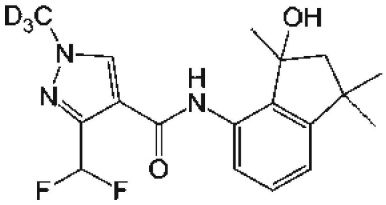



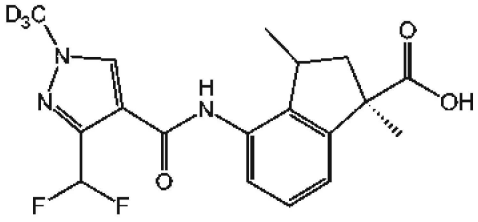
1'-COOH-S-2840-B (MW = 363.4)

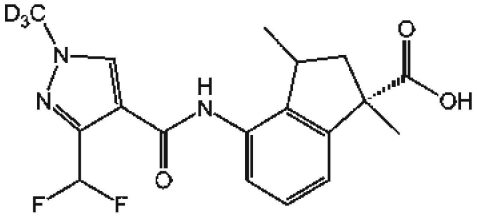
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid

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

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

Reference Standard	Analytical Standard Number	% Purity	Expiration Date
S-2399	2375a	95.3	05OCT16
3'-OH-S-2840	2379a	99.7	02JUN17
1'-COOH-S-2840-A	2393a	100	03FEB18
1'-COOH-S-2840-B	2394a	99.6	04FEB18

TEST SYSTEM

The test system used for the validation was untreated control sediment from study VP-38970, entitled "Aquatic Field Dissipation of S-2399 Following Foliar Application of S-2399 2.84 SC to a Flooded Rice Field in Louisiana." The sediment was stored in a freezer (ca. -20°C) when not in use.

1.2 EQUIPMENT AND REAGENTS

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

1.2.1 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 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)
Graduated cylinder, stoppered (100 mL)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), Pasteur, volumetric and/or automatic (volumes of 0.20 to 20 mL)
Reciprocating mechanical shaker, (Erbach or equivalent)
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 20 mL, 1g Waters # 186000117 or equivalent)
Storage containers, polypropylene, 125-mL with caps (Cole Parmer # 06041-12)
Volumetric flasks, (**pre-rinsed with methanol**) assorted volumes as needed

1.2.2 REAGENTS

Acetic acid, reagent grade or equivalent
Acetone, reagent grade or equivalent
Ammonium Acetate, reagent grade or equivalent
Hydrochloric acid, 12N, pesticide quality or equivalent
Methanol, pesticide quality or equivalent
Sodium acetate anhydrous (or sodium acetate trihydrate), reagent grade or equivalent
Water, HPLC grade

EXPERIMENTAL PROCEDURES

1.3 STANDARD SOLUTIONS PREPARATION

Stock, working calibration and fortification solutions were prepared as per method. Stock standard solutions were prepared from the neat reference standards for use in the preparation of fortification solutions and instrument calibration solutions. Although the choice of measuring device (pipet, volumetric flask, etc) and quantities measured are not restricted by method RM-50S-1, the 1 mg/mL standard solutions were prepared using automatic pipettors and 22-mL glass vials. Standard solutions with concentrations at 10 µg/ mL were prepared using an automatic pipettor and volumetric flasks. Standard solutions with concentrations at 1 µg/mL and below were prepared using an automatic

pipettor or glass pipets and volumetric flasks. All standard solutions were stored refrigerated (ca. 4°C) when not in use.

The calibration standards were validated according to Valent SOP VR-003-09 "Analytical Standard Solutions".

Individual 1 mg/mL internal standard stock solutions (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), were prepared by adding approximately 12 mg of neat compound to a respective 22-mL glass vial. Solutions were created by pipetting the appropriate amount of methanol to ensure a 1.0 mg/mL solution. The stock solutions were stored refrigerated (ca. 4°C) when not in use.

A 1 µg/mL internal standard solution containing all four internal standards was prepared by pipetting 100 µL of each individual 1 mg/mL internal standard stock solution into a 100-mL volumetric flask and diluting to volume with methanol. The solution was transferred into a glass container with a Teflon-lined cap and stored refrigerated (ca. 4°C) when not in use.

A 2 µg/L internal standard solution containing all four internal standards was prepared by pipetting 2.0 mL of the 1 µg/mL internal standard solution into a 1000-mL volumetric flask and diluting to volume with methanol: water (1:1, v:v). The solution was transferred into a glass container with a Teflon-lined cap and stored refrigerated (ca. 4°C) when not in use.

Three 2-L solutions each containing all four internal standards at a concentration of 1 µg/L solution were prepared by pipetting 2.0 mL of the 1 µg/mL internal standard solution into a 2000-mL volumetric flask and diluting to volume with methanol: water (1:1, v:v). Solutions were transferred into glass containers and stored refrigerated (ca. 4°C) when not in use.

1.4 SAMPLE PREPARATION

All samples were prepared as per the method. Analytical sets consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified at the LOQ (0.010 µg/g), and five untreated controls fortified at 10×LOQ (0.100 µg/g).

1.5 SAMPLE ANALYSIS

All samples were analyzed as per the method. The specific instrumentation and settings for method validation are listed below.

1.5.1 INSTRUMENTATION

An Agilent Technologies 1200 series HPLC with tandem Applied Biosystems API 4000 mass selective detector, with turbo-ion spray ionization in positive ion (for S-2399) and negative ion (for 1'-COOH-S-2840-A, 1'-COOH-S-2840-B and 3'-OH-S-2840) modes was used for sample analysis.

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 = 10mM ammonium acetate in HPLC water
	B = 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 = 10.5 min, 10% A + 90% B T = 14.5 min, 10% A + 90% B T = 15.0 min, 65% A + 35% B T = 19.0 min, 65% A + 35% B
Flow Rate Program:	700 µL/min
Injection Volume:	25 µL

Typical MS-MS Parameters:

Period 1: 1'-COOH-S-2840-A (retention time ca. 5.8 min) and 1'-COOH-S-2840-B (ca. 6.3 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 2: 3'-OH-S-2840 (retention time ca. 9.0 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 3: S-2399 (retention time ca. 9.4 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

This analytical method validation report has been amended to incorporate modifications based on the recommendations of the independent laboratory validation study (Reference 1). Method RM-50S-2 is a modified version of Valent analytical method RM-50S-1. In this modified version, notes are provided in section 5, ANALYTICAL PROCEDURES during steps 3 and 4 of the method to facilitate analyte percent recoveries. In step 3, centrifugation is recommended (at typical rate and time) if a precipitate is observed during the pH adjustment with buffer solution. In step 4, additional 5-ml rinses with methanol are encouraged to ensure that a complete transfer of the analytes will occur. The final analyte dilution solvent was also clarified as being methanol/HPLC water (1/1,v/v) when an internal standard solution was not in use. If optional internal standards are used, samples are diluted with the 2 µg/L ISFV (Internal Standard Final Volume) solution [instead of methanol/HPLC water (1/1, v/v)]. Minor modifications were included in the method for clarification. All analytical methods are presented in APPENDIX 2.

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 ratio $Area_{Analyte}$ / $Area_{Internal\ standard}$ were entered into an EXCEL® spreadsheet to calculate the data.

To calculate the line (curve) for instrument calibration, the peak area ratio and the nominal concentration of each of the calibration standards were input into an Excel spreadsheet. A weighted linear standard curve ($Y=aX+b$) was generated for the analytical set, and the coefficients (*a* and *b*) of the line (curve) were determined. The line (curve) was used to calibrate the instrument, determine the acceptability of the standard injections and to calculate the sample analyte concentrations. The line (curve) was generated by plotting the standard detector response (area ratio) versus the nominal standard concentration and was weighted relative to the largest standard concentration. Six different standard concentrations were injected in the analytical set.

Calibration Standard	Relative Weighting Calcn (High Std Conc / Std Conc)	Number of Entries in Data Set
10 µg/L	10 / 10	1
5 µg/L	10 / 5	2
2.5 µg/L	10 / 2.5	4
1 µg/L	10 / 1	10
0.5 µg/L	10 / 0.5	20
0.25 µg/L	10 / 0.25	40

The concentrations (µg/L) of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840- B detected in sample extracts were interpolated from the respective standard calibration lines (curves). Analyte concentrations for the standards were calculated by the Excel spreadsheet using the equation:

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

where: $C_{standard}$: concentration of analyte in the standard solution, (µg/L)
a: slope
b: y intercept
Detector response: $Area\ ratio_{STD}$

The analyte concentrations in the sample were calculated as follows:

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX + b] \times EV \times C \times D}{AV \times E}$$

where: X = Sample response (peak area or area ratio)
a = slope
b = intercept
C = Final volume (0.001 L)
EV= Eluant Volume (100 mL)
AV = Aliquot volume (0.5 mL)
D = Dilution factor (1)
E = Sample weight (10 g)

Percent recoveries for the fortified sam ples were corrected for the average detector response observed in the associated control samples using the average peak area ratio observed in the control samples. The average detector response of the control sa mples 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 validation.

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 S-2399 fortified untreated control sediment (sample Ft 1), in set VP-39207-1, fortified at 0.010 µg/g, is as follows:

Corrected detector response: peak area ratio Ft 1 – average peak area ratio in controls UTC1 and UTC2 = 0.6396-[(0.1048+0.1083)/2] = 0.6396 – 0.10655 = 0.53305

$$Conc\ Ft1 = \frac{[(8.99E-01)(0.53305) - 8.27E-02] \text{ x } 100 \text{ x } 0.001 \text{ x } 1}{0.5 \text{ x } 10}$$

$$= \frac{0.3966 \text{ x } 100 \text{ x } 0.001 \text{ x } 1}{5}$$

Concentration of Ft 1(µg/g) = 0.00793 (79.3% recovery)

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

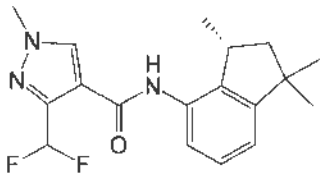
1. INTRODUCTION

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.

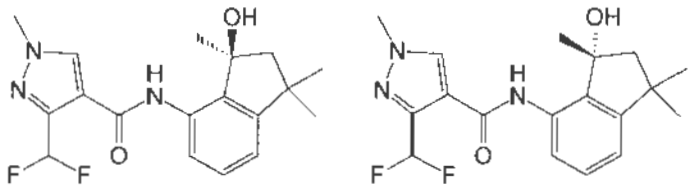
Briefly, the residues are extracted from soil using two extractions of acetone/water (4/1, v/v) followed by an extraction with acetone/0.5M HCl (4/1, v/v). The residues are partitioned into dichloromethane. The dichloromethane from this step is evaporated, and the residues are quantitated by LC/MS/MS.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

2. MATERIALS

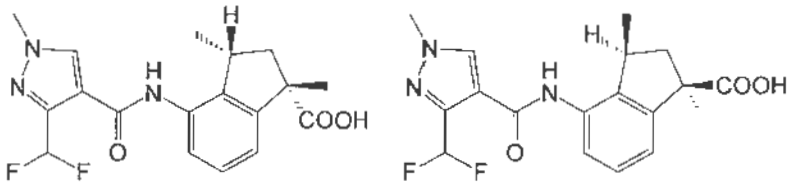
2.1. ANALYTICAL REFERENCE STANDARDS



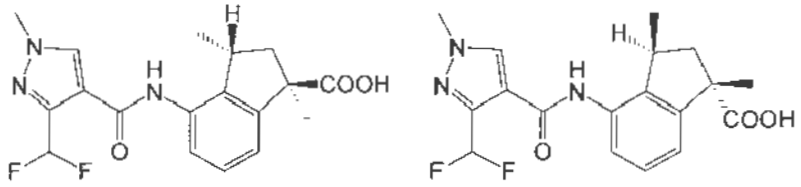
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3'*R*)-1',1',3'-trimethyl-2',3'-dihydro-1'*H*-inden-4'-yl]-1*H*-pyrazole-4-carboxamide



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

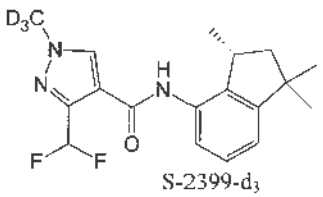


1`-COOH-S-2840-A (MW 363.4)
4´-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1´R,3´S)-1´,3´-dimethyl-2´,3´ dihydro-1´*H*-indene-1´-carboxylic acid
4´-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1´S,3´R)-1´,3´-dimethyl-2´,3´ dihydro-1´*H*-indene-1´-carboxylic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

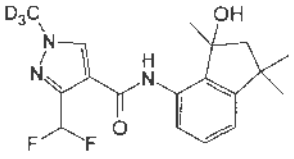


1`-COOH-S-2840-B (MW - 363.4)
4´-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1´R,3´R)-1´,3´-dimethyl-2´,3´ dihydro-1´*H*-indene-1´-carboxylic acid
4´-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1´S,3´S)-1´,3´-dimethyl-2´,3´ dihydro-1´*H*-indene-1´-carboxylic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

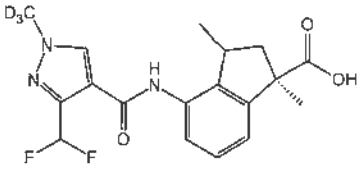
2.2. OPTIONAL INTERNAL STANDARDS



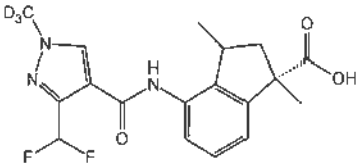
S-2399-d_3



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 proportioas 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 abuve 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 equivalent

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 1µg/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:

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

$$\mu g/L = (6.690\ E\text{-}11x\ 4{,}272 \times 4{,}272) + 1.144\ E\text{-}04x\ 4.272) - 2.161\ E\text{-}03 = 0.488$$

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:

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

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:

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

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:

$$\mu g/L = (6.690\ E\text{-}11x\ (4{,}272\text{-}332) \times (4{,}272\text{-}332)) + (1.144\ E\text{-}04x\ (4{,}272\text{-}332)) - 2.161\ E\text{-}03 = 0.450$$

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:

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

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:

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

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

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 n 0.00025 ug/mL standard in the calibration.

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

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

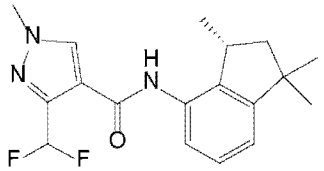
1. INTRODUCTION

This method determines residues of S-2399 and metabolites 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in sediment and soil. Each S-2840 metabolite includes enantiomers. For the carboxylic acid metabolites, the A and B designations of the acids are based on their isomeric similarities. 1'-COOH-S-2840-A has two enantiomers and 1'-COOH-S-2840-B also contains two enantiomers; however, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B are diastereomers. This method is a modification of Valent method RM-50S. Briefly, the residues are extracted from sediment or 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 combined extract is adjusted to pH ca. = 5 and undergoes solid phase extraction (SPE) utilizing an Oasis HLB 20cc (1 g) cartridge. Analytes are eluted and sample eluent total volumes are adjusted to 100 mL using methanol. Samples are reconstituted in 1:1 (v/v) methanol: water (with or without internal standard) and analyzed using high-performance liquid chromatography with tandem mass spectrometry LC/MS-MS (with turbo-ion spray ionization in positive and negative ion modes).

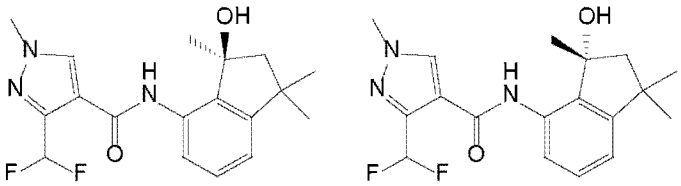
2. MATERIALS

2.1 Analytical Reference Standards

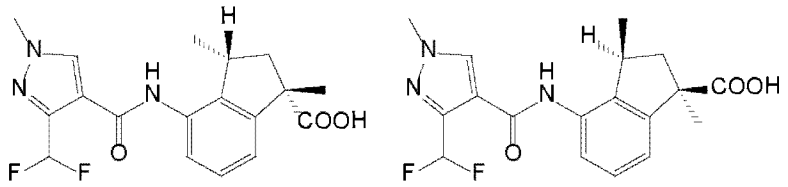
The following analytical reference standards are used:



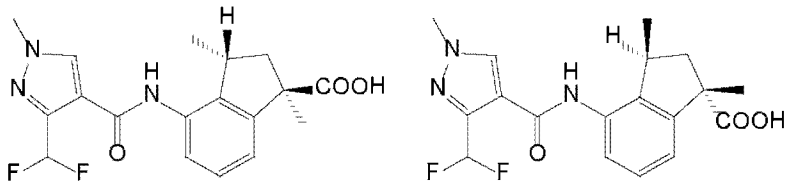
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3′*R*)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1*H*-pyrazole-4-carboxamide



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

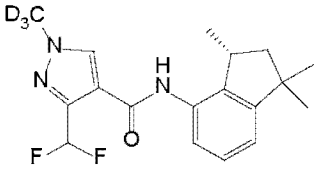


1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

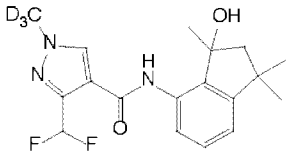


1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

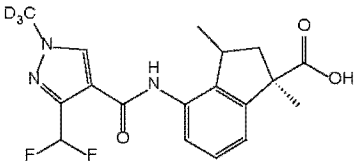
2.2 Optional Internal Standards



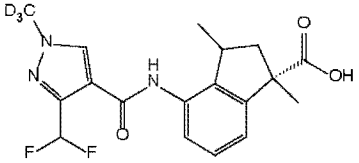
S-2399-d_3



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

Below are examples for preparing standards. Additional dilutions and/or alternate concentrations may be prepared to generate appropriate standards. Other volumes (aliquots and final volumes) may be prepared and other containers and measuring devices (*e.g.*, vials and pipets) may be used as long as proportions are maintained and the preparation is 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 the final volume using a pipet 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.

Intermediate 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 intermediate 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 calibration 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 calibration 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 calibration 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 calibration 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 calibration 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, the calibration standards prepared above have volumes diluted with the 1 µg/L Internal Standard Final Volume Solution [instead of methanol/HPLC water (1/1, v/v)].

Store the calibration standard solutions in a refrigerator or freezer when not in use.

2.3.1 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 and/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, 2 µg/L:

Transfer a 2.0 mL aliquot of the 1 µg/mL Intermediate Internal Standard 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.

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.

2.4 Reagents

Acetic acid, reagent grade or equivalent
Acetone, reagent grade or equivalent
Ammonium Acetate, reagent grade or equivalent
Hydrochloric acid, 12N, pesticide quality or equivalent
Methanol, pesticide quality or equivalent
Sodium acetate anhydrous (or sodium acetate trihydrate), reagent grade or equivalent
Water, HPLC grade

2.5 Reagent Solution Preparation

Reagent solutions may be prepared in the following manner. Other volumes (and measuring devices) 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.

10mM Ammonium Acetate in HPLC Water
Add 0.77g ammonium acetate into 1 L of HPLC water.

1M acetic acid in HPLC Water
Add 28.6 mL of concentrated acetic acid into a 500-mL volumetric flask containing some HPLC water. Fill the flask to volume with HPLC water. Mix well, transfer to a reagent bottle (as necessary), and store at room temperature.

1M sodium acetate in HPLC Water
Add 41.0 g of anhydrous sodium acetate (or 68.0 g of sodium acetate trihydrate) into a 500-mL volumetric flask containing some HPLC water. Swirl and sonicate to dissolve solid. Fill the flask to volume with HPLC water. Mix well, transfer to a reagent bottle (as necessary), and store at room temperature.

Acetic acid/Sodium acetate buffer, 1M
Add 180 mL of 1M acetic acid solution and 320 mL of 1M sodium acetate into a 500-mL glass bottle. Mix well. Verify the pH of the solution (pH 5). Store at room temperature.

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.

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)

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)
Graduated cylinder, stoppered (100 mL)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), Pasteur, volumetric and/or automatic (volumes of 0.20 to 20 mL)
Reciprocating mechanical shaker, (Erbach or equivalent)
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 1g Waters # 186000117 or equivalent)
Storage containers, polypropylene, 125-mL with caps (Cole Parmer # 06041-12)
Volumetric flasks, (**pre-rinsed with methanol**) assorted volumes as needed

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. Conditions shown below 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.

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 = 10mM ammonium acetate in HPLC water B = 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 = 10.5 min, 10% A + 90% B T = 14.5 min, 10% A + 90% B T = 15.0 min, 65% A + 35% B T = 19.0 min, 65% A + 35% B
Flow Rate Program:	700 µL/min
Injection Volume:	25 µL

Typical MS-MS Parameters:

Period 1: 1'-COOH-S-2840-A (retention time ca. 5.8 min) and 1'-COOH-S-2840-B (ca. 6.3 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 2: 3'-OH-S-2840 (retention time ca. 9.0 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 3: S-2399 (retention time ca. 9.4 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

5 ANALYTICAL PROCEDURES

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

1. 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 Section 11 NOTES).

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

2. 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 4000 rpm or as needed to separate the solids from the extraction solvent. Decant the sample extract into a 125-mL polypropylene (or other suitable) container.

Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample. Disintegrate the pellet, if needed and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes and decant the sample extract into the 125-mL polypropylene (or other suitable) container 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. Disintegrate the pellet, if needed and shake on reciprocating shaker for 30 minutes. **Immediately** centrifuge the sample for approximately 5 minutes and decant the sample extract into the 125-mL polypropylene (or other suitable) container containing the first two extracts. **Immediately** add 2 mL of 0.5 M sodium acetate solution to the 125-mL polypropylene (or other suitable) container and mix well.

3. pH Adjustment

   Adjust the pH of the samples to ca. pH = 5 by pipetting 2 mL of 1 M acetic acid / sodium acetate buffer into each sample. Mix well.

4. SPE Cartridge Conditioning, Loading and Extraction

   Condition an Oasis HLB 1 g, 20cc SPE cartridge by adding ~10 mL of methanol followed by ~20 mL of HPLC-grade water under gentle vacuum.

   Load a portion of the sample onto the cartridge. Collect all eluent in a 50-mL polypropylene tube. Continue adding portions of the sample onto the cartridge and collect all eluent. Once the volume in the 50-mL polypropylene tube reaches ca. 40 mL, transfer the contents to a stoppered 100-mL graduated cylinder.

   Continue adding the sample onto the cartridge and collecting all eluent until the entire combined extract sample has been extracted and all eluent has been collected in the 50-mL polypropylene tube and transferred to the stoppered 100-mL graduated cylinder.

   Rinse the 125-mL polypropylene (or other suitable) container with two ca. 5-mL aliquots of methanol and add each rinse to the SPE cartridge. Collect all eluent in the 50-mL polypropylene tube. Transfer the contents to the stoppered 100-mL graduated cylinder. Rinse the 50-mL polypropylene tube with ca. 5 mL of methanol and add the rinse to the graduated cylinder. Adjust the eluent volume to 100 mL using methanol. Stopper the cylinder and mix well. Transfer a portion of the solution to a suitable storage container for future analyses, if needed.

5. Final Analyte Solution Preparation and Injection

   Pipet 0.5 mL of the eluent and 0.5 mL of the 2 µg/L ISFV (Internal Standard Final Volume) solution into an autosampler vial. Cap and mix well. Inject ca. 25 µL onto the LC-MS/MS for analysis.

   Note: If optional internal standards are not used, replace the 2 µg/L Internal Standard Final Volume Solution with methanol/HPLC water (1/1, v/v).

A set of 24 samples will require approximately 4 hours of preparation for LC-MS/MS analysis. Each sample will run for approximately 19.5 minutes on the LC-MS/MS. The total time for the complete analysis of 24 samples is 12 hours.

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 linear calibration or 2^{nd}-order polynomial calibration, analyze a minimum of five calibration standard concentrations within the analytical sequence. A typical set of standards includes concentrations of 0.50, 1, 2.5, 5, 10 and the required 0.25 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 peak area 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 1 µg/L for linear or 2^{nd}-order polynomial calibrations) are also analyzed as part of the analytical sequence. Typically, the sequence is constructed with the following order: a continuing calibration standard, 1 to 6 prepared samples, a continuing calibration standard or a calibration standard, 1 to 6 prepared samples, and a continuing calibration standard. The sequence must begin and end with a continuing calibration standard. With the calibration standard (analyzed for the curve fit) included, this ensures a minimum of three continuing calibration standard responses for evaluation. The coefficient of variation (CV) of the continuing calibration standard calculated concentrations 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 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 detector response obtained is within the calibrated response range of the LC/MS-MS.

7 CALCULATIONS

To calculate the line or curve for instrument calibration, the peak area (or peak area ratio) and the concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to a linear regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte peak area ([or peak area ratio] or expressed as Peak Units; e.g., as area/10^6). 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
10 µg/L	10 / 10	1
5 µg/L	10 / 5	2
2.5 µg/L	10 / 2.5	4
1 µg/L	10 / 1	10
0.5 µg/L	10 / 0.5	20
0.25 µg/L	10 / 0.25	40

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

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX + b] \text{ x EV x C x D}}{\text{AV x E}}$$

where:
X = Sample response (peak area or area ratio)
a = slope
b = intercept
C = Final volume (0.001 L)
EV= Eluant Volume (100 mL)
AV = Aliquot volume (0.5 mL)
D = Dilution factor (1)
E = Sample weight (10 g)

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

$$\text{Sample Concentration, } (\mu g/L) = \frac{[aX^2 + bX + c] \text{ x EV x C x D}}{\text{AV x E}}$$

where:
X = Sample response (peak area or area ratio)
a = constant (for x^2 term in polynomial fit)
b = constant (for x term in polynomial fit)
c = constant (for intercept in polynomial fit)
C = Final volume (0.001 L)
EV= Eluant Volume (100 mL)
AV = Aliquot volume (0.5 mL)
D = Dilution factor (1)
E = Sample weight (10 g)

For calculation of analyte recovery in a fortified sample, the recovery is corrected by using either the peak units (peak area or area ratio) or the concentration observed in the control sample. If the

peak units in the control sample are 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 peak units in the control sample are subtracted from the peak units 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 response 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:

$$\text{Coefficient of Variation, \%} = \frac{\text{Standard Deviation, calculated concentration}}{\text{Average Response, calculated concentration}} \text{ x } 100\%$$

8 LIMIT OF DETECTION

The limit of detection (LOD) of this method is 0.005 ppm. The detection limit is based on a 10-g sample weight, a 100-mL extract/eluant volume, 0.5 mL aliquot volume, 1 mL final volume, a 1x dilution, and a 0.25 µg/L calibration standard (as the lowest concentration in the set of calibration standards):

$$\text{Limit of Detection} = \frac{0.001 \text{ L Final Vol. x } 100 \text{ mL x } 0.25 \text{ µg/L Stnd}}{0.5 \text{ mL aliquot x } 10 \text{ g sample}} = 0.005 \text{ µg/g}$$

9 LIMIT OF QUANTIFICATION

This method has a limit of quantification (LOQ) of 0.010 µg/g, for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in sediment and soil.

10 CHROMATOGRAMS

Example chromatograms are shown in Figures 1 through 16.

11 NOTES

Fortified control samples are to be analyzed with each set of samples. Method recoveries must be 70 to 120% to be acceptable, unless approved by the supervising chemist responsible for the analysis, or by the Study Director. 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.

1. INTRODUCTION

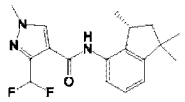
Method RM-50S-2 is a modified version of Valent analytical method RM-50S-1. In this modified version, notes are provided in section 5 ANALYTICAL PROCEDURES during steps 3 and 4 to facilitate analyte percent recoveries. In step 3, centrifugation is recommended if a precipitate is observed during the pH adjustment with buffer solution. In step 4, additional 5-ml rinses with methanol are allowed to ensure that a complete transfer of the analytes has occurred. The final analyte dilution solvent was also clarified as being methanol/HPLC water (1/1,v/v) when an internal standard solution was not in use. If optional internal standards are used, samples are diluted with the 2 µg/L ISFV (Internal Standard Final Volume) solution [instead of methanol/HPLC water (1/1, v/v)]. RM-50S-2 is valid via RM-50S method validation as no changes were made that impact the extractability of the analytical method. The analytical data (including chromatograms) in method RM-50S-2 are from the original validation of RM-50S.

This method determines residues of S-2399 and metabolites 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in sediment and soil. Each S-2840 metabolite includes enantiomers. For the carboxylic acid metabolites, the A and B designations of the acids are based on their isomeric similarities. 1'-COOH-S-2840-A has two enantiomers and 1'-COOH-S-2840-B also contains two enantiomers; however, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B are diastereomers. This method is a modification of Valent method RM-50S. Briefly, the residues are extracted from sediment or 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 combined extract is adjusted to pH ca. = 5 and undergoes solid phase extraction (SPE) utilizing an Oasis HLB 20cc (1 g) cartridge. Analytes are eluted and sample eluent total volumes are adjusted to 100 mL using methanol. Samples are reconstituted in 1:1 (v/v) methanol: water (with or without internal standard) and analyzed using high-performance liquid chromatography with tandem mass spectrometry LC/MS-MS (with turbo-ion spray ionization in positive and negative ion modes).

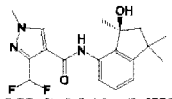
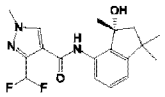
2. MATERIALS

2.1 Analytical Reference Standards

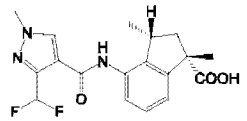
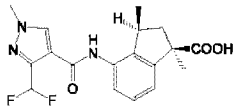
The following analytical reference standards are used:



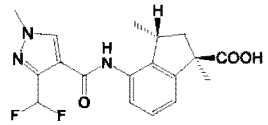
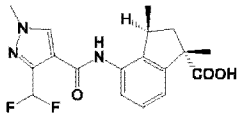
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3′*R*)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1*H*-pyrazole-4-carboxamide

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

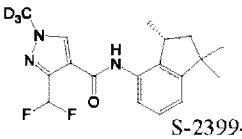



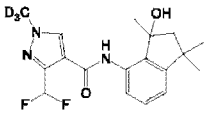
_1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid

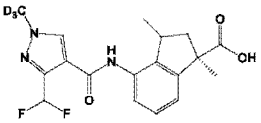
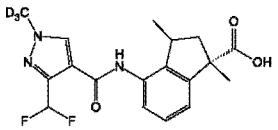



1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

2.2 Optional Internal Standards


S-2399-d_3


3'-OH-S-2840-d_3

2.3 Analytical Reference Standard Preparation

Below are examples for preparing standards. Additional dilutions and/or alternate concentrations may be prepared to generate appropriate standards. Other volumes (aliquots and final volumes) may be prepared and other containers and measuring devices (*e.g.*, vials and pipets) may be used as long as proportions are maintained and the preparation is 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 the final volume using a pipet 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.

Intermediate 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 intermediate 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 calibration 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 calibration 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 calibration 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 calibration 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 calibration 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, the calibration standards prepared above have volumes diluted with the 1 µg/L Internal Standard Final Volume Solution [instead of methanol/HPLC water (1/1, v/v)].

Store the calibration standard solutions in a refrigerator or freezer when not in use.

2.3.1 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 and/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, 2 µg/L:

Transfer a 2.0 mL aliquot of the 1 µg/mL Intermediate Internal Standard 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.

Internal Standard Final Volume Solution, 1 µg/L:

Transfer a 1.0 mL aliquot of the 1 µg/mL Intermediate Internal Standard 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.

2.4 Reagents

Acetic acid, reagent grade or equivalent
Acetone, reagent grade or equivalent
Ammonium Acetate, reagent grade or equivalent
Hydrochloric acid, 12N, pesticide quality or equivalent
Methanol, pesticide quality or equivalent
Sodium acetate anhydrous (or sodium acetate trihydrate), reagent grade or equivalent
Water, HPLC grade

2.5 Reagent Solution Preparation

Reagent solutions may be prepared in the following manner. Other volumes (and measuring devices) 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.

10mM Ammonium Acetate in HPLC Water
Add 0.77g ammonium acetate into 1 L of HPLC water.

1M acetic acid in HPLC Water
Add 28.6 mL of concentrated acetic acid into a 500-mL volumetric flask containing some HPLC water. Fill the flask to volume with HPLC water. Mix well, transfer to a reagent bottle (as necessary), and store at room temperature.

1M sodium acetate in HPLC Water
Add 41.0 g of anhydrous sodium acetate (or 68.0 g of sodium acetate trihydrate) into a 500-mL volumetric flask containing some HPLC water. Swirl and sonicate to dissolve solid. Fill the flask to volume with HPLC water. Mix well, transfer to a reagent bottle (as necessary), and store at room temperature.

Acetic acid/Sodium acetate buffer, 1M
Add 180 mL of 1M acetic acid solution and 320 mL of 1M sodium acetate into a 500-mL glass bottle. Mix well. Verify the pH of the solution (pH 5). Store at room temperature.

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.

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)

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)
Graduated cylinder, stoppered (100 mL)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), Pasteur, volumetric and/or automatic (volumes of 0.20 to 20 mL)
Reciprocating mechanical shaker, (Erbach or equivalent)
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 1g Waters # 186000117 or equivalent)
Storage containers, polypropylene, 125-mL with caps (Cole Parmer # 06041-12)
Volumetric flasks, (**pre-rinsed with methanol**) assorted volumes as needed

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. Conditions shown below 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.

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 = 10mM ammonium acetate in HPLC water B = 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 = 10.5 min, 10% A + 90% B T = 14.5 min, 10% A + 90% B T = 15.0 min, 65% A + 35% B T = 19.0 min, 65% A + 35% B
Flow Rate Program:	700 µL/min
Injection Volume:	25 µL

Typical MS-MS Parameters:

Period 1: 1'-COOH-S-2840-A (retention time ca. 5.8 min) and 1'-COOH-S-2840-B (ca. 6.3 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 2: 3'-OH-S-2840 (retention time ca. 9.0 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

Period 3: S-2399 (retention time ca. 9.4 min)

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

*Values in parentheses are for qualifier / confirmatory ions.

5 ANALYTICAL PROCEDURES

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

1. 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 Section 11 NOTES).

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

2. 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 4000 rpm or as needed to separate the solids from the extraction solvent. Decant the sample extract into a 125-mL polypropylene (or other suitable) container.

Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample. Disintegrate the pellet, if needed and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes and decant the sample extract into the 125-mL polypropylene (or other suitable) container 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. Disintegrate the pellet, if needed and shake on reciprocating shaker for 30 minutes. **Immediately** centrifuge the sample for approximately 5 minutes and decant the sample extract into the 125-mL polypropylene (or other suitable) container containing the first two extracts. **Immediately** add 2 mL of 0.5 M sodium acetate solution to the 125-mL polypropylene (or other suitable) container and mix well.

3. pH Adjustment

   Adjust the pH of the samples to ca. pH = 5 by pipetting 2 mL of 1 M acetic acid / sodium acetate buffer into each sample. Mix well.

   Note: If a precipitate is observed once the buffer is added, then centrifugation (ca. 5 minutes at approximately 4000 rpm or as needed) is recommended prior to going to Step 4.

4. SPE Cartridge Conditioning, Loading and Extraction

   Condition an Oasis HLB 1 g, 20cc SPE cartridge by adding ~10 mL of methanol followed by ~20 mL of HPLC-grade water under gentle vacuum.

   Load a portion of the sample onto the cartridge. Collect all eluent in a 50-mL polypropylene tube. Continue adding portions of the sample onto the cartridge and collect all eluent. Once the volume in the 50-mL polypropylene tube reaches ca. 40 mL, transfer the contents to a stoppered 100-mL graduated cylinder.

   Continue adding the sample onto the cartridge and collecting all eluent until the entire combined extract sample has been extracted and all eluent has been collected in the 50-mL polypropylene tube and transferred to the stoppered 100-mL graduated cylinder.

   Rinse the 125-mL polypropylene (or other suitable) container with two ca. 5-mL aliquots of methanol and add each rinse to the SPE cartridge.

   Note: Additional (ca. two) 5-mL methanol rinses of the 125-mL polypropylene (or other suitable) container may be required to achieve adequate recoveries.

   Collect all eluent in the 50-mL polypropylene tube. Transfer the contents to the stoppered 100-mL graduated cylinder. Rinse the 50-mL polypropylene tube with ca. 5 mL of methanol and add the rinse to the graduated cylinder. Adjust the eluent volume to 100 mL using methanol. Stopper the cylinder and mix well. Transfer a portion of the solution to a suitable storage container for future analyses, if needed.

5. Final Analyte Solution Preparation and Injection

   Pipet 0.5 mL of the eluent and 0.5 mL of the 2 µg/L ISFV (Internal Standard Final Volume) solution into an autosampler vial. Cap and mix well. Inject ca. 25 µL onto the LC-MS/MS for analysis.

   Note: If optional internal standards are not used, replace the 2 µg/L Internal Standard Final Volume Solution with methanol/HPLC water (1/1, v/v).

A set of 24 samples will require approximately 4 hours of preparation for LC-MS/MS analysis. Each sample will run for approximately 19.5 minutes on the LC-MS/MS. The total time for the complete analysis of 24 samples is 12 hours.

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 linear calibration or 2^{nd}-order polynomial calibration, analyze a minimum of five calibration standard concentrations within the analytical sequence. A typical set of standards includes concentrations of 0.50, 1, 2.5, 5, 10 and the required 0.25 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 peak area 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 1 µg/L for linear or 2^{nd}-order polynomial calibrations) are also analyzed as part of the analytical sequence. Typically, the sequence is constructed with the following order: a continuing calibration standard, 1 to 6 prepared samples, a continuing calibration standard or a calibration standard, 1 to 6 prepared samples, and a continuing calibration standard. The sequence must begin and end with a continuing calibration standard. With the calibration standard (analyzed for the curve fit) included, this ensures a minimum of three continuing calibration standard responses for evaluation. The coefficient of variation (CV) of the continuing calibration standard calculated concentrations must be 15% or less for the analytical set to be acceptable, unless approved by the supervising chemist or Study Director.

If the peak area observed for a sample is greater than the peak area of the highest calibration standard, the sample extract must be diluted with methanol/HPLC water (1/1,v/v) when an internal standard solution is not in use. If optional internal standards are used and the peak area ratio observed for a sample is greater than the peak area ratio of the highest calibration standard, the sample is diluted with the 2 µg/L ISFV (Internal Standard Final Volume) solution [instead of

methanol/HPLC water (1/1, v/v)]. The sample extract must be diluted such that the detector response obtained is within the calibrated response range of the LC/MS-MS.

7 CALCULATIONS

To calculate the line or curve for instrument calibration, the peak area (or peak area ratio) and the concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to a linear regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte peak area ([or peak area ratio] or expressed as Peak Units; e.g., as area/10^6). 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
10 µg/L	10 / 10	1
5 µg/L	10 / 5	2
2.5 µg/L	10 / 2.5	4
1 µg/L	10 / 1	10
0.5 µg/L	10 / 0.5	20
0.25 µg/L	10 / 0.25	40

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

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX + b] \times EV \times C \times D}{AV \times E}$$

where:
X = Sample response (peak area or area ratio)
a = slope
b = intercept
C = Final volume (0.001 L)
EV= Eluant Volume (100 mL)
AV = Aliquot volume (0.5 mL)
D = Dilution factor (1)
E = Sample weight (10 g)

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

$$\text{Sample Concentration, } (\mu g/g) = \frac{[aX^2 + bX + c] \times EV \times C \times D}{AV \times E}$$

where:
X = Sample response (peak area or area ratio)
a = constant (for x^2 term in polynomial fit)
b = constant (for x term in polynomial fit)

c = constant (for intercept in polynomial fit)
C = Final volume (0.001 L)
EV= Eluant Volume (100 mL)
AV = Aliquot volume (0.5 mL)
D = Dilution factor (1)
E = Sample weight (10 g)

For calculation of analyte recovery in a fortified sample, the recovery is corrected by using either the peak units (peak area or area ratio) or the concentration observed in the control sample. If the peak units in the control sample are 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 peak units in the control sample are subtracted from the peak units in the fortified sample prior to calculating a corrected concentration. This corrected concentration is then used to calculate percent recovery:

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

For evaluation of the continuing calibration standards (with a minimum of three interspersed within the analytical sequence), the average response 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:

$$\text{Coefficient of Variation, \%} = \frac{\text{Standard Deviation, calculated concentration}}{\text{Average Response, calculated concentration}} \times 100\%$$

8 LIMIT OF DETECTION

The limit of detection (LOD) of this method is 0.005 ppm. The detection limit is based on a 10-g sample weight, a 100-mL extract/eluant volume, 0.5 mL aliquot volume, 1 mL final volume, a 1x dilution, and a 0.25 µg/L calibration standard (as the lowest concentration in the set of calibration standards):

$$\text{Limit of Detection} = \frac{0.001\text{L Final Vol. x 100 mL x 0.25 µg/L Stnd}}{0.5\text{ mL aliquot x 10 g Sample}} = 0.005\ \mu g/g$$

9 LIMIT OF QUANTIFICATION

This method has a limit of quantification (LOQ) of 0.010 µg/g, for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in sediment and soil.

REPORT AMENDMENT

PROTOCOL NUMBER: VP-39207

AMENDMENT NUMBER: 1

REPORT TITLE: S-2399: Validation of Valent Method RM-50S-1, "Determination of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Sediment and Soil"

TEST SUBSTANCE: S-2399

ORIGINAL REPORT DATE: May 19, 2016

AMENDED REPORT DATE: See title page.

REASON FOR AMENDMENT: This analytical method validation report has been amended to incorporate modifications based on the recommendations of the independent laboratory validation study. Method RM-50S-2 is a modified version of Valent analytical method RM-50S-1. In this modified version, notes are provided in section 5 ANALYTICAL PROCEDURES during steps 3 and 4 of the method to facilitate analyte percent recoveries. The final analyte dilution solvent was also clarified as being methanol/HPLC water (1/1,v/v) when an internal standard solution was not in use. If optional internal standards are used, samples are diluted with the 2 µg/L ISFV (Internal Standard Final Volume) solution [instead of methanol/HPLC water (1/1, v/v)]. Minor modifications were included in the method for clarification.

IMPACT ON STUDY: The amendment did not affect the integrity of the study data and has no impact on the study conclusions.

DESCRIPTION OF AMENDMENT:

General – The company name has been updated throughout the report from Corporation to LLC.
Page 7, 14-15 & 17. Added a statement that centrifugation is recommended if a precipitate is observed during the pH adjustment process and that additional rinses are encouraged to ensure a complete transfer of the analytes in steps 3 and 4 of the analytical method. Also included a clarifying statement regarding the final analyte dilution solutions. Other minor changes were made to the method to enhance the clarity of the text.
Pages 7, 14, 17 & 18. The reference to the independent laboratory validation was added.
Page 17. Corrected the Valent analytical method identifier from RM-49S-1 to RM-50S-1.
Page 26. Protocol amendment 2 was added to the report.
Pages 28- 55 (Appendix 2). The original analytical method, RM-50S, has been added to the validation report.
Pages 87- 118 (Appendix 2). The modified version of the analytical method, RM-50S-2, has been added to the validation report.
Page 89. The analytical method has a comment stating that the method has been updated and includes the items that are being amended.

Page 89. A comment was added to the modified analytical method, RM-50S-2, stating that RM-50S-2 is valid via RM-50S method validation as no other changes were made that impact the extractability of the analytical method.
Page 91. Commas were removed from section 2.3 Analytical Reference Standard Preparation, Calibration Standard Solutions (0.25 µg/L – 10 µg/L inclusive) for grammar.
Page 92. Commas were removed from section 2.3.1 Optional Internal Standard Preparation, internal standard solutions (1 µg/mL, 2 µg/L & 1 µg/L inclusive) for grammar.

Page 92. For the preparation of the 1 µg/L Internal Standard Final Volume Solution, the name of the 1 µg/mL Intermediate Internal Standard Solution was corrected from Intermediate Internal Stock Solution.
Page 97. Added an update in section 5 ANALYTICAL PROCEDURES during step 3 of the analytical method that centrifugation is recommended (ca. 5 minutes at approximately 4000 rpm or as needed)) if a precipitate is observed during the pH adjustment with buffer solution.
Page 97. Added an update in section 5 ANALYTICAL PROCEDURES during step 4 of the analytical method that additional (ca. two) 5-ml rinses with methanol are encouraged to ensure that a complete transfer of the analytes has occurred.
Pages 98-99. A clarifying statement regarding the final analyte dilution solutions was added.
Page 99. The units in the polynomial sample concentration equation were corrected from µg/L to µg/g.
Page 125. The page numbers in the Index of Chromatograms has been updated.
Page 213 – 215 (Appendix 6). The report amendment was added.

REPORT AMENDMENT SIGNATURES:

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Residue Chemist
Valent Technical Center
Valent U.S.A. LLC

Testing Facility Manager: _____ Date: 16-Aug-2019

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