SUMMARY

This report has been amended due to the analytical method, RM-50W, being inadvertently submitted to the EPA as the analytical method validation report. In addition, a modification was required for RM-50W based on the recommendations of the independent laboratory validation study (Moate).
The method, RM-50W-1, was created to specify that the HPLC column, an Agilent Eclipse XDB-C8 column, may not be substituted. 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 modifications (including method modifications) are documented in the report amendment (APPENDIX 6).

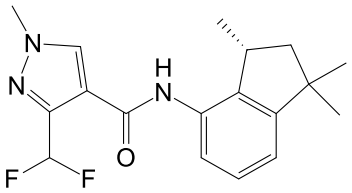
The validation of residue analytical method RM-50W, entitled "S-2399: Validation of Valent Method RM-50W, Determination of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Surface Water", was completed. This method has a limit of detection (LOD) of 0.5 µg/L, and a limit of quantification (LOQ) of 1 µg/L, for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in surface water. The protocol for this method validation is included in APPENDIX 1. Method RM-50W (including sample calculations and example chromatograms) as well as the modified version of the method, RM-50W-1 are presented in APPENDIX 2

This method was validated by fortification of untreated source water 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 1 µg/L (LOQ) and 10 µg/L (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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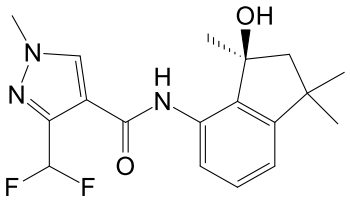

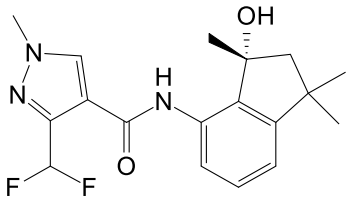

3'-OH-S-2840 (MW = 349.4)

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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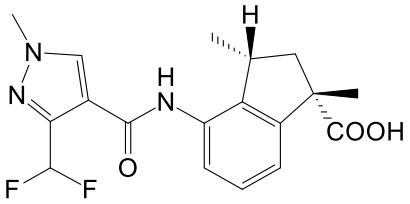
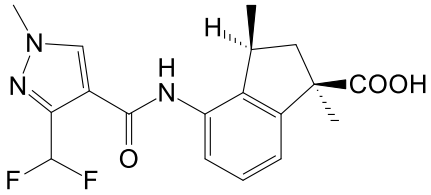
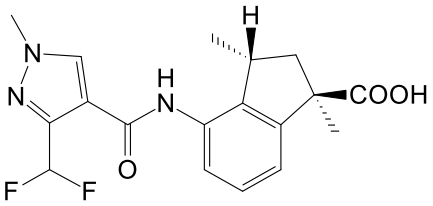
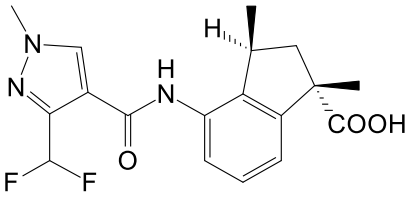




1'-COOH-S-2840-A (MW = 363.4)

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

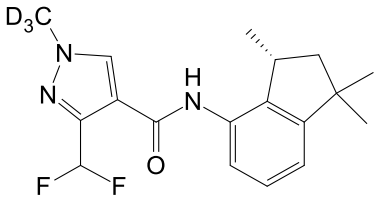




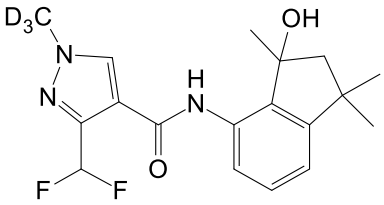
1'-COOH-S-2840-B (MW = 363.4)

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

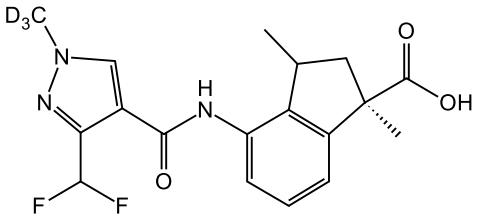
2.1 Internal Standards



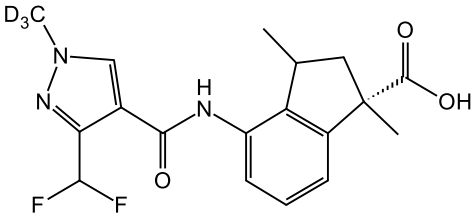
S-2399-d_3



3'-OH-S-2840-d_3



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



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

The 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	96.0	11OCT15
3'-OH-S-2840	2379a	99.7	02JUN17
1'-COOH-S-2840-A	2393a	100	12FEB16
1'-COOH-S-2840-B	2394a	99.6	12FEB16

1.2 TEST SYSTEM

The test system used for the validation was untreated source water 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 water was stored in a freezer (ca. -20°C) when not in use.

1.3 EQUIPMENT AND REAGENTS

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

1.3.1 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading
Centrifuge tubes, polypropylene, 15 mL graduated (Accuflow #EK-4020)
Freezer, -20°C capable
Glass bottles, 4 oz amber with Teflon-coated caps (VWR Cat. No. 36319-435)
Glass vials (approximately 60 mL)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series)
Mass Spectrometer (Applied Biosystems API 4000)
Pipette(s), automatic - capable of accurately dispensing volumes of 0.20 to 20 mL
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 500 mg Waters # 186000116)
Volumetric flasks, (**pre-rinsed with methanol**) assorted volumes

1.3.2 REAGENTS

Acetic acid, reagent grade
Ammonium Acetate, reagent grade
Methanol, pesticide quality
Sodium acetate anhydrous, reagent grade
Water, HPLC grade

EXPERIMENTAL PROCEDURES

1.4 STANDARD SOLUTIONS PREPARATION

Stock, working calibration, fortification solutions and internal standards were prepared as per method. Stock standard and internal 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-50W, the 1 mg/mL standard and internal standard solutions were prepared using an automatic pipettor and 22-mL glass vials. The 10 µg/mL standard solution and both 1 µg/mL solutions (one with internal standard added and one without) were prepared using an automatic pipettor and volumetric flasks. The 1 ug/mL fortification solution (without internal standard), 1 ug/L internal standard solution, and the working calibration standard solutions (except the 0.25 ug/L) were all prepared in twice the volume than what was outlined in the method.

The 1 ug/mL intermediate solution used to generate the calibration standards (internal standard present), was created by pipetting 100 uL of each 1 mg/mL analyte stock solution and bringing the total volume up to 100 mL using the 1 ug/L internal standard solution. The solvent used for the 1 ug/L internal standard solution was methanol: water (1:1 v/v). All subsequent calibration standards made from the 1 ug/mL intermediate solution used the 1 ug/L internal standard solution as the diluent instead of methanol: water (1:1 v/v) All standard solutions were stored refrigerated (ca. 4°C) when not in use.

The calibration standards were not validated according to Valent SOP VR-003-09 "Analytical Standard Solutions". Typically, identical concentrations of working and monitoring calibration standard solutions are made by diluting identical concentration working and monitoring analyte stock solutions. Monitoring solutions were not created below the 1 µg/mL concentration. The 1 µg/mL working and monitoring solutions (without internal standard) were used to validate the preparation of the fortification solutions. The preparation of the working calibration standard solutions was verified using the coefficient of determination and back-calculated concentrations of the standard curve.

1.5 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 (1.0 µg/L), and five untreated controls fortified at 10×LOQ (10.0 µg/L).

1.6 SAMPLE ANALYSIS

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

1.6.1 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 = 5mM 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 = 12.5 min, 10% A + 90% B T = 13.0 min, 65% A + 35% B T = 17.0 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 report was amended due to the analytical method, RM-50W, being inadvertently submitted to the EPA as the analytical method validation report. In addition, a modification was required for RM-50W based on the recommendations of the independent laboratory validation study (Moate). The method, RM-50W-1, was created to specify that the HPLC column, an Agilent Eclipse XDB-C8 column, may not be substituted. 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 standa rds 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. The modified method, RM-50W-1 is presented in APPENDIX 2.

Valent residue analytical method RM-50W, entitled S-2399: "Determination of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Surface Water" was evaluated with a limit of detection (LOD) of 0.5 µg/L, and a limit of quantification (LOQ) of 1.0 µg/L. The protocol for this method validation is included in APPENDIX 1, and the methods are presented in APPENDIX 2. Example chromatograms are presented in APPENDIX 4.

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 each analyte with each set of analyses, 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 within each 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:

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

where: X = Sample response (area ratio)
a = slope
b = intercept
C = Final volume (10 mL)
D = Dilution factor (4)
E = Sample volume (20 mL)

Percent recoveries for the fortified samples 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 samples 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 surface water (sample Ft 1), in set V-15-39100-4, fortified at 1.0 µg/L, is as follows:

Corrected detector response: peak area ratio Ft 1 – average peak area ratio in controls UTC1 and UTC2

= 0.5452-[(0.0342+0.0396)/2] = 0.5452 – 0.0369 = 0.5083

$$Conc\ Ft\,1 = \frac{[1.07E\text{-}00\ (0.5083)\ - 5.61E\text{-}02] \text{ x } 10 \text{ x } 4}{20}$$

$$= \frac{0.487781 \text{ x } 40}{20}$$

Concentration of Ft 1(µg/L) = 0.975562 (97.6% recovery)

The calculated value in the residue raw data spreadsheet was 97.8%. The difference in calculated percent recovery is believed to be due to rounding.

CONCLUSION

This report was amended due to the analytical method, RM-50W, being inadvertently submitted to the EPA as the analytical method validation report. In addition, a modification was required for RM-50W based on the recommendations of the independent laboratory validation study (Moate). The modified method, RM-50W-1, was created to specify that the HPLC column, an Agilent Eclipse XDB-C8 column, may not be substituted. 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 modifications (including method modifications) are documented in the report amendment (APPENDIX 6).

Valent residue analytical method RM-50W, entitled S-2399: "Determination of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Surface Water", was successfully validated. This method was validated by fortification of untreated control surface water with S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B at nominal concentrations of 1.0 µg/L (LOQ) and 10.0 µg/L (10xLOQ), and has a limit of detection (LOD) of 0.5 µg/L.

REFERENCE

1. Moate, T. (2017). S-2399: Independent Laboratory Validation of Valent U.S.A. Corporation's Residue Analytical Method for the Determination of S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in Surface Water (Method Number: RM-50W). Fresno, CA. Golden Pacific Laboratories, LLC, (MRID: 49706429).

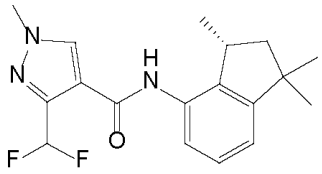
1. INTRODUCTION

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 surface water. 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 involves the solid phase extraction (SPE) of 20 mL of surface water utilizing an Oasis HLB 12cc (500mg) cartridge. Samples are adjusted to pH = 5 using 1M acetic acid / sodium acetate buffer solution and loaded onto SPE cartridges. Samples are eluted with methanol. Sample eluent total volumes are adjusted to 10 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).

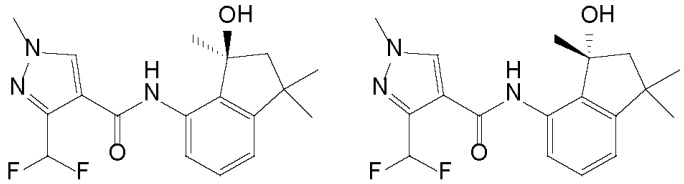
2. MATERIALS

2.1 Analytical Reference Standards

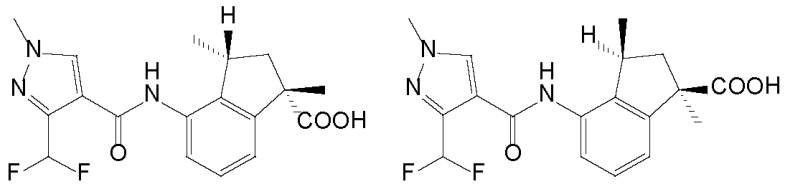
The following analytical reference standards are used:



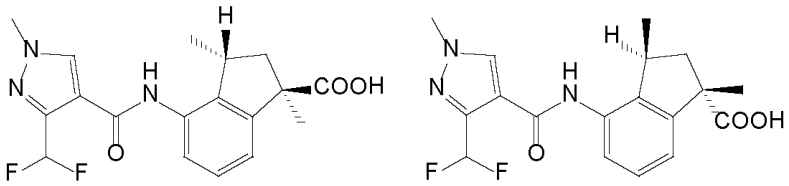
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3′*R*)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1*H*-pyrazole-4-carboxamide



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

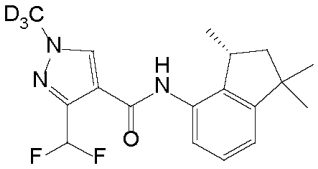


1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

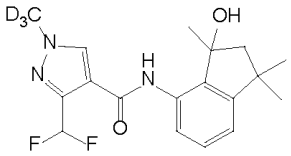


1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

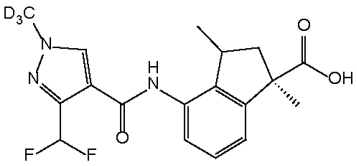
2.2 Optional Internal Standards



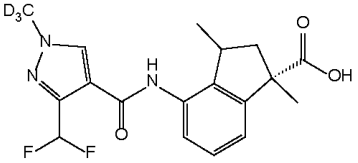
S-2399-d_3



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 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.

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.

Fortification Solution, 0.1 ug/mL (Prepared Fresh Daily):

Transfer 1.0 mL aliquot of the 1 ug/mL fortification solution into a vial. Pipette 9 mL of acetone and mix. As this solution is prepared daily, validation of this fortification solution is not required.

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, 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 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
Ammonium Acetate, reagent grade or equivalent
Methanol, pesticide quality or equivalent
Sodium acetate anhydrous, 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.

5mM Ammonium Acetate in HPLC Water
Add 0.385g 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.

3 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading
Centrifuge tubes, polypropylene, 15 mL graduated (Accuflow #EK-4020 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 and 60 mL or equivalent)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), automatic - capable of accurately dispensing volumes of 0.20 to 20 mL
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 500 mg Waters # 186000116 or equivalent)
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 = 5mM 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 = 12.5 min, 10% A + 90% B T = 13.0 min, 65% A + 35% B T = 17.0 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

1. Sample Setup

Mix the bulk water sample well making sure not to spill the contents. Pipet 20 mL of sample into a suitable glass container. If needed, allow the bulk water samples to thaw enough to draw a 20-mL sample. At this point, a control sample to be used for method recoveries may be fortified using the appropriate standard solution. Note: Samples may be pipetted into 60-mL glass vials and stored frozen (ca.-20°C) prior to fortification and analysis.

2. Acidification

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

3. SPE Cartridge Conditioning and Loading

Condition an Oasis HLB 500 mg, 12cc SPE cartridge by adding ~5 mL of methanol followed by ~10 mL of HPLC-grade water under gentle vacuum. Load the sample onto the cartridge. Pipet 1.7 mL of HPLC-grade water onto the cartridge. Discard the eluent. Pipet 9 mL of methanol into each 60-mL glass vial and swirl the vial. Add the methanol rinse of the 60-mL vial to the respective SPE cartridge. Collect the methanol eluent in a graduated 15-mL polypropylene tube. Adjust the final volume to 10 mL using methanol. Cap the vial and mix well.

4. Final Analyte Solution Preparation and Injection

Pipet 0.25 mL of the methanol eluent, 0.25 mL of HPLC-grade water and 0.5 mL of the 2 µg/L ISFV (Internal Standard Final Volume) solution into an autosampler vial. Mix well. **Note**: If internal standard is not used, pipet 0.5 mL of methanol/HPLC water (1/1, v/v) instead. Inject 25 µL onto the LC-MS/MS for analysis.

A set of 24 samples will require approximately 4 hours of preparation for LC-MS/MS analysis. Each sample will run for approximately 17.5 minutes on the LC-MS/MS. The total time for the complete analysis of 24 samples is 11 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 2nd-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 2nd-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 responses 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 and the diluted extract analyzed. The sample extract must be diluted such that the peaks obtained are within the calibrated response range of the LC/MS-MS.

7 CALCULATIONS

To calculate the line or curve for instrument calibration, the peak area and the concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to either a linear or a 2nd-order polynomial regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte peak area (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:

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

where: X = Sample response (peak area or area ratio)
a = slope
b = intercept
C = Final volume (0.010 L)
D = Dilution factor (4)
E = Sample volume (0.020 L)

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

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

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 slope in polynomial fit)
C = Final volume (0.010 L)
D = Dilution factor (4)
E = Sample volume (0.020L)

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 0.0005 ppm. The detection limit is based on a 20-mL sample volume, a 10-mL final volume, a 4x dilution, and a 0.25 µg/L calibration standard (as the lowest concentration in the set of calibration standards):

$$\text{Limit of Detection} = \frac{0.010\text{L Final Vol. x } 4 \text{ x } 0.25\,\mu\text{g/L Stnd}}{0.020\text{L Sample Vol.}} = 0.5\,\mu\text{g/L}$$

9 LIMIT OF QUANTIFICATION

This method has a limit of quantification (LOQ) of 1.0 µg/L (LOQ), for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in surface water

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.

1. INTRODUCTION

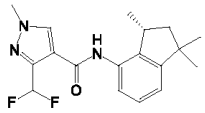
Method RM-50W-1 is a modified version of Valent analytical method RM-50W. In this modified version, it is specified that the HPLC column may not be substituted. The final analyte dilution solvent was also clarified as being methanol/HPLC water (1/1,v/v) when an internal standard solution was not in use. 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-50W-1 is valid via RM-50W method validation as no other changes were made that affect the extractability of the analytical method. The analytical data (including chromatograms) in method RM-50W-1 are from the original validation of RM-50W.

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 surface water. 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 involves the solid phase extraction (SPE) of 20 mL of surface water utilizing an Oasis HLB 12cc (500mg) cartridge. Samples are adjusted to pH = 5 using 1M acetic acid / sodium acetate buffer solution and loaded onto SPE cartridges. Samples are eluted with methanol. Sample eluent total volumes are adjusted to 10 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).

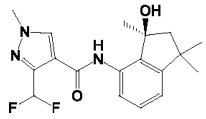
2. MATERIALS

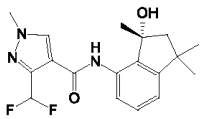
2.1 Analytical Reference Standards

The following analytical reference standards are used:

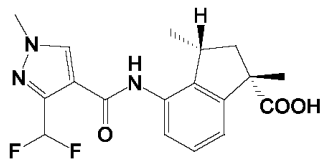
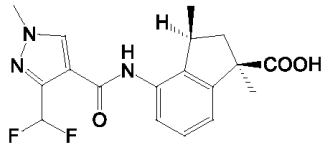


S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3′*R*)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1*H*-pyrazole-4-carboxamide

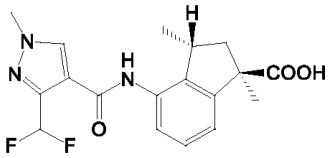
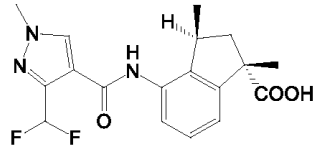




3'-OH-S-2840 (MW = 349.4)
3-(Difluoromethyl)-*N*-[3′-hydroxy-(3′R)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1-methyl-1*H*-pyrazole-4-carboxamide
3-(Difluoromethyl)-*N*-[3′-hydroxy-(3′S)-1′,1′,3′-trimethyl-2′,3′-dihydro-1′*H*-inden-4′-yl]-1-methyl-1*H*-pyrazole-4-carboxamide

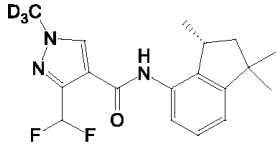



1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

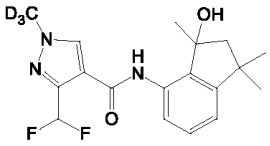



1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

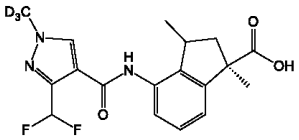
2.2 Optional Internal Standards



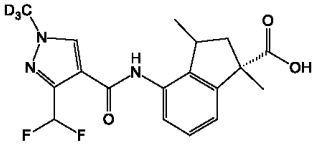
S-2399-d_3



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 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.

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.

Fortification Solution, 0.1 ug/mL (Prepared Fresh Daily):

Transfer 1.0 mL aliquot of the 1 ug/mL fortification solution into a vial. Pipette 9 mL of acetone and mix. As this solution is prepared daily, validation of this fortification solution is not required.

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, 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 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
Ammonium Acetate, reagent grade or equivalent
Methanol, pesticide quality or equivalent
Sodium acetate anhydrous, 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.

5mM Ammonium Acetate in HPLC Water
Add 0.385g 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.

3 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading
Centrifuge tubes, polypropylene, 15 mL graduated (Accuflow #EK-4020 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 and 60 mL or equivalent)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), automatic - capable of accurately dispensing volumes of 0.20 to 20 mL
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 500 mg Waters # 186000116 or equivalent)
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. **The Agilent Eclipse XDB-C8 column may not be substituted.** Other conditions shown below are suggested for this analysis. The conditions may be modified as needed (except for HPLC column) 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 **Note:** This column may not be substituted.
Column Oven Temperature:	40 ± 1°C
Mobile Phase:	A = 5mM 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 = 12.5 min, 10% A + 90% B T = 13.0 min, 65% A + 35% B T = 17.0 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

1. Sample Setup

Mix the bulk water sample well making sure not to spill the contents. Pipet 20 mL of sample into a suitable glass container. If needed, allow the bulk water samples to thaw enough to draw a 20-mL sample. At this point, a control sample to be used for method recoveries may be fortified using the appropriate standard solution. Note: Samples may be pipetted into 60-mL glass vials and stored frozen (ca.-20°C) prior to fortification and analysis.

2. Acidification

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

3. SPE Cartridge Conditioning and Loading

Condition an Oasis HLB 500 mg, 12cc SPE cartridge by adding ~5 mL of methanol followed by ~10 mL of HPLC grade water under gentle vacuum. Load the sample onto the cartridge. Pipet 1.7 mL of HPLC-grade water onto the cartridge. Discard the eluant. Pipet 9 mL of methanol into each 60-mL glass vial and swirl the vial. Add the methanol rinse of the 60-mL vial to the respective SPE cartridge. Collect the methanol eluent in a graduated 15-mL polypropylene tube. Adjust the final volume to 10 mL using methanol. Cap the vial and mix well.

4. Final Analyte Solution Preparation and Injection

Pipet 0.25 mL of the methanol eluent, 0.25 mL of HPLC-grade water and 0.5 mL of the 2 µg/L ISFV (Internal Standard Final Volume) solution into an autosampler vial. Mix well. Note: If internal standard is not used, pipet 0.5 mL of methanol/HPLC water (1/1,v/v) instead. Inject 25 µL onto the LC-MS/MS for analysis.

A set of 24 samples will require approximately 4 hours of preparation for LC-MS/MS analysis. Each sample will run for approximately 17.5 minutes on the LC-MS/MS. The total time for the complete analysis of 24 samples is 11 hours.

6 LC/MS-MS ANALYSIS

Instrument calibration is performed using either a linear fit with a non-zero intercept or a 2nd-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 2nd-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 2nd-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 responses 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 and the concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to either a linear or a 2nd-order polynomial regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte peak area (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:

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

where: X = Sample response (peak area or area ratio)
a = slope
b = intercept
C = Final volume (0.010 L)
D = Dilution factor (4)
E = Sample volume (0.020 L)

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

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

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 slope in polynomial fit)
C = Final volume (0.010 L)
D = Dilution factor (4)
E = Sample volume (0.020 L)

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 0.0005 ppm. The detection limit is based on a 20-mL sample volume, a 10-mL final volume, a 4x dilution, and a 0.25 µg/L calibration standard (as the lowest concentration in the set of calibration standards):

$$\text{Limit of Detection} = \frac{0.010\text{L mL Final Vol. x 4 x 0.25 µg/L Stnd}}{0.020\text{L Sample Vol.}} = 0.5\ \mu g/L$$

9 LIMIT OF QUANTIFICATION

This method has a limit of quantification (LOQ) of 1.0 µg/L for S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B in surface water.

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.

REPORT AMENDMENT

PROTOCOL NUMBER: VP-39100

AMENDMENT NUMBER: 1

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

TEST SUBSTANCE: S-2399

ORIGINAL REPORT DATE: May 19, 2016

AMENDED REPORT DATE: See title page.

REASON FOR AMENDMENT: The analytical method, RM-50W, was inadvertently submitted to the EPA as the analytical method validation report. In addition, a modification was required for RM-50W based on the recommendations of the independent laboratory validation study. The modified method, RM-50W-1, was created to specify that the HPLC column, an Agilent Eclipse XDB-C8 column, may not be substituted. 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 made to 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 & 17. Added a statement that the HPLC column may not be substituted. 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. The reference to the independent laboratory validation was added.
Page 12. The word "fortification" was added for clarification in section 1.4 STANDARD SOLUTIONS PREPARATION to update the sentence to read " The 1 µg/mL working and monitoring solutions (without internal standard) were used to validate the preparation of the fortification solutions."
Page 24. Protocol amendment 2 was added to the report.
Page 25. Protocol amendment 3 was added to the report.
Pages 58 - 88 (Appendix 2). The modified analytical method, RM-50W-1, has been added to the validation report in addition to analytical method RM-50W.
Page 61. The analytical method has a comment stating that the method has been amended and includes the items that are being amended.

Page 62. A comment was added to the modified analytical method, RM-50W-1, stating that RM-50W-1 is valid via RM-50W method validation as no other changes were made to the analytical method.
Page 62. A comment was added to the modified analytical method, RM-50W-1, stating that the analytical data (including chromatograms) in method RM-50W-1 are from the original validation of RM-50W.
Page 65. 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 66. It is specified that the HPLC column may not be substituted.
Page 69. A clarifying statement regarding the final analyte dilution solution was added.
Page 184 – 186 (Appendix 6). The report amendment was added.

REPORT AMENDMENT SIGNATURES:

Study Director: _____ Date: 14 AUG 2019

James Foster, PhD
Residue Chemist
Valent Technical Center
Valent U.S.A. LLC

Testing Facility Manager: _____ Date: 14-AUG-2019

Eric Tamichi
Director, Regulatory
Valent Technical Center
Valent U.S.A. LLC