# I. SUMMARY

Valent U.S.A. Corporation contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to conduct an Independent Laboratory Validation. The objective of this study was to validate the analytical method (provided by Valent U.S.A. Corporation) entitled "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). Valent U.S.A. Corporation requested that the method be validated for all analytes. The method was successfully validated using Liquid Chromatography (LC) equipped with tandem mass spectrometer (MS/MS) detector. The analysis was validated for the determination of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in bottled spring water during the first method trial. The analytical method was validated to demonstrate method ruggedness and to meet US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 Test Guidelines requirements for environmental chemistry methods and associated independent laboratory validation. The study was conducted under EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160.

**Independent Laboratory Validation**

One control sample was used in this study. The water sample, Crystal Geyser® Alpine Spring Water®, was obtained from a local grocery store. There was no response in the control matrix samples in the chromatograms corresponding to the retention time of the analytes.

The control water sample was analyzed using the provided analytical method. Water samples were buffered with an acetic acid/sodium acetate solution and taken through solid phase extraction (SPE) cleanup. Samples were eluted with methanol and extracts were then diluted with methanol, to a known volume. Final extracts were diluted with HPLC-grade water and an internal standard solution, vialed, and analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

The method was validated at 1 and 10 µg/L (ng/mL) for the detection of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in surface water.

# II. MATERIALS

## A. Equipment

The equipment that was used is listed below:

- Analytical balance: Mettler Toledo XS204
- Top Loading balance: Mettler Toledo MS3002S/03
- Top Loading balance: Mettler Toldedo PB3002-S
- Volumetric flasks, glass: various sizes
- Bottles, amber glass with Teflon lined cap: various sizes (including 30 mL)
- Volumetric glass pipettes: various sizes
- Polypropylene tubes: BD Falcon 15 mL
- Graduated cylinders: various volumes
- Graduated mixing cylinders: various volumes
- Micropipette, Drummond Wiretrol disposable: various volumes
- Disposable Pasteur pipettes, glass
- Repeating pipette: Eppendorf Stream
- pH test strips (BDH, 0-14)
- HPLC/GC vials and caps: 1.8 mL
- SPE cartridges: Waters Oasis LB, SPE column, 500 mg (Cat# 186000116)
- SPE Manifold: Burdick & Jackson (24 position)
- LC-MS/MS: AB Sciex API4000 LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP controller, and SIL-20AC HT autosampler
- HPLC Column: Agilent Eclipse XDB-C8, 5-µm, 150 x 4.6 mm (Cat# 993967-906)

## B. Reagents and Standards

The following chemicals were used:

| Chemical | Distributer | Grade | Part No: |
|---|---|---|---|
| Acetic Acid | Fisher | ACS | A38S |
| Acetonitrile | Fisher | Optima® | A996 |
| Ammonium Acetate | Fisher | HPLC | A639 |
| Methanol | VWR | ChromAR® | MK304110 |
| Sodium Acetate | VWR | ACS | 97061-994 |
| Water | Fisher | HPLC | W5-4 |

Preparation of Reagent Solutions:

*1 M Sodium Acetate in Water:* Prepared by adding 41 g sodium acetate to approximately 400 mL of HPLC-grade water in a 500-mL mixing cylinder. The sodium acetate was dissolved and the solution was brought up to volume (500 mL) with HPLC-grade water and mixed well.

*1 M Acetic Acid Solution:* Prepared by adding 28.6 mL of acetic acid to approximately 400 mL of HPLC-grade water into a 500-mL mixing cylinder and mixed well. The solution was brought up to volume (500 mL) with HPLC-grade water and mixed well.

*1 M Acetic Acid/1M Sodium Acetate Buffer Solution (pH ~5)*: Prepared by adding 180 mL of 1 M acetic acid to approximately 320 mL of 1 M sodium acetate solution into a 500-mL mixing cylinder and mixed well. Resulting pH checked with pH test strips.

*Methanol/water (50:50, v/v)*: Prepared by adding 500 mL of methanol to 500 mL of HPLC-grade water and mixing well.

*5 mM Ammonium Acetate in Water:* Prepared by adding 0.38 g ammonium acetate to approximately 800 mL of HPLC-grade water in a 1-L volumetric flask. The ammonium acetate was dissolved and the solution was brought up to volume (1000 mL) with HPLC-grade water and mixed well.

*Needle-Wash, acetonitrile/water (50:50, v/v)*: Prepared by combining 500 mL of acetonitrile with 500 mL of HPLC-grade water and mixing well.

1. Reference Substances

   The S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B analytical reference standards and deuterated internal standards (IS) were received in good condition on June 22, 2016 from Valent Technical Center, Dublin, CA. The certificate of analysis for each of the standards is in the archives at GPL. The following table contains detailed information for the analytical standard used in this study.

| Analytical Standard | Lot # | Purity (%) | Expiration Date |
|---|---|---|---|
| S-2399 | AS 2375a | 95.3 | 10/03/2018 |
| 3′-OH-S-2840 | AS 2397b | 97.7 | 09/13/2017 |
| 1′-COOH-S-2840-A | AS 2393b | 99.8 | 02/18/2018 |
| 1′-COOH-S-2840-B | AS 2394b | 99.5 | 02/16/2018 |
| S-2399-$d_3$ | AS 2422a | 97.2 | 12/28/2020 |
| 3′-OH-S-2840-$d_3$ | AS 2414a | 99.3 | 11/18/2020 |
| 1′-COOH-S-2840-A-$d_3$ | AS 2420a | 99.9 | 12/21/2020 |
| 1′-COOH-S-2840-B-$d_3$ | AS 2421a | 99.5 | 12/21/2020 |

Upon receipt, the neat reference standards were stored in a freezer set to maintain ≤ -10 °C (frozen).

2. Preparation of Standard Solutions

The S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B analytical reference substances were used in the preparation of the fortification and calibration solutions. All concentrations listed are nominal values. All standard solutions were stored frozen when not in use. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

**a. Stock Solutions**

To prepare individual stock solutions approximately 10 mg of the reference substances were weighed directly into individual 10-mL volumetric flasks and diluted to the mark with acetone. After correcting for purity, the stock solution contained approximately 1 mg/mL of each reference substance.

To prepare individual stock solutions of the internal standards (IS) approximately 10 mg of the IS reference substances were weighed directly into individual 10-mL volumetric flasks and diluted to the mark with methanol. After correcting for purity, the stock solution contained approximately 1 mg/mL of each reference substance.

**b. Fortification Solutions**

***Intermediate Solution***

One mL of each 1 mg/mL stock solution was diluted to a 100-mL final volume in acetone to prepare a mixed intermediate standard solution at 10 µg/mL.

***High Fortification Solution***

Ten mL of the 10 µg/mL intermediate solution was diluted to a 100-mL final volume in acetone to prepare a high fortification solution at 1 µg/mL.

***Low Fortification Solution***

One mL of the 1 µg/mL high fortification solution was diluted to a 10-mL final volume in acetone to prepare a low fortification solution at 0.1 µg/mL.

**c. Internal Standard (IS) Solutions**

***Intermediate IS Solution***

A volume of 0.2 mL of each 1 mg/mL IS stock solution was diluted to a 200-mL final volume in methanol to prepare a mixed solution at 1 µg/mL.

***Calibration Standard IS Solution***

One mL of the 1 µg/mL intermediate IS solution was diluted to a 1000-mL final volume in methanol/HPLC-water (50:50, v/v) to prepare an external standard dilution solution at 1 ng/mL.

***Sample IS Solution***

A volume of 0.2 mL of the 1 µg/mL intermediate IS solution was diluted to a final volume of 100 mL in methanol/HPLC-water (50:50, v/v).to prepare an IS sample extract solution at 2 ng/mL.

**d. Calibration Solutions**

Calibration solutions were prepared by serial dilution starting with the 1 µg/mL high fortification solution. All dilutions were made using the 1 ng/mL Calibration Standard IS as the diluent. Nominal

concentrations of the calibration standards appear in the table below.

| Parent Solution (ng/mL) | Aliquot Volume (mL) | Final Volume (mL) | Final Concentration (ng/mL) |
|---|---|---|---|
| 1000 | 1 | 100 | 10 |
| 10 | 50 | 100 | 5 |
| 5 | 50 | 100 | 2.5 |
| 2.5 | 40 | 100 | 1 |
| 1 | 50 | 100 | 0.5 |
| 0.5 | 50 | 100 | 0.25 |

### C. Safety and Health

Material Safety Data Sheets (MSDS) and/or Safety Data Sheets (SDS) were available. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. Caution should be used when handling concentrated acetic acid. There were no other procedural steps that required special precautions in order to avoid safety or health hazards.

## III. METHODS

### A. Principle of Analytical Method

The analysis for the determination of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in bottled spring water was performed according to the reference method titled "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). The limit of quantitation (LOQ) was 1.0 µg/L for all analytes. The method defined limit of detection LOD was 0.5 µg/L. A copy of the analytical method is in Appendix B.

The method samples were validated as set 698ILV01 on January 23, 2017. All samples were successfully validated in one trial. The set consisted of one reagent blank sample, two control samples, seven LOQ laboratory fortification samples fortified at 1.0 µg/L and five 10x LOQ laboratory fortification samples fortified at 10 µg/L. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number, the sample set designation and a sample number (e.g., 698ILV01-1).

Sub-samples (20 mL) of control water were fortified. Samples were buffered to pH ~5 with acetic acid/sodium acetate buffer. Samples were taken through a solid phase extraction cleanup through the use of an Oasis SPE cartridge. The resulting eluent was brought up to a known volume in methanol. Sample extracts were vialed

in water and IS solution for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

## B. Analytical Procedure

### 1. Control Matrix

The water control matrix sample (Crystal Geyser #03 1228161154) was purchased from a local store on January 23, 2017. The sample was stored frozen when not in use. The bottled water report was obtained from the manufacturer and is presented in Appendix C.

### 2. Preparation of Samples

Sub-samples (20 mL) of the control water matrix were measured into 30-mL amber glass bottles.

### 3. Fortifications

Independent laboratory validation samples were fortified at the LOQ (1 µg/L) or 10x the LOQ (10 µg/L). Fortifications were completed as follows:

| Fortification Level | Amount and Concentration of Spiking Solution Used |
|---|---|
| LOQ (1 µg/L) | 200 µL of a 0.1 µg/mL Low Fortification solution |
| 10x LOQ (10 µg/L) | 200 µL of a 1 µg/mL High Fortification solution |

### 4. Extraction

After fortification, 2 mL of 1 M acetic acid/sodium acetate buffer solution was added to each sample. Each sample jar was capped and mixed well by hand. SPE (Oasis) cartridges were conditioned with 5 mL of methanol followed by equilibration using 10-mL of HPLC-grade water. Gentle vacuum was used needed for all SPE steps. Entire sample was loaded onto the SPE column and the cartridge washed with 1.7 mL of HPLC-grade water. Solvent was discarded for all steps. A 15-mL plastic centrifuge tube was then placed under each SPE cartridge. Sample bottles were rinsed with 9 mL of methanol and added to the SPE columns for elution of the analytes. The methanol eluent was collected in the centrifuge tubes and the volume was brought up to 10 mL with methanol.

An aliquot of 0.25 mL of sample was combined with 0.25 mL HPLC-grade water and 0.5 mL of 2 ng/mL IS sample solution into autosampler vials. The vials were capped and mixed well prior to analysis by LC-MS/MS.

## C. Instrumentation

Instrument: AB Sciex API4000 LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC HT Autosampler

HPLC Column: Agilent Eclipse XDB-C8, 5 µm, 150 x 4.6 mm
Part # 993967-906
Serial # USKR080489

Guard Column: Phenomenex Security Guard Cartridge C8

Column Heater: Hot Sleeve 539157, 40 °C

Data System: Analyst Chromatography Data System version 1.6, AB Sciex

Mobile Phases:

A) Methanol
B) 5 mM Ammonium Acetate in Water

Flow Rate: 0.7 mL/minute
Run Time: 17.0 minutes
Injection Volume: 25 µL
Gradient Program:

| Time (minutes) | %A | %B |
|---|---|---|
| 0.0 | 35 | 65 |
| 1.0 | 35 | 65 |
| 6.0 | 90 | 10 |
| 7.0 | 65 | 35 |
| 10.0 | 65 | 35 |
| 10.5 | 90 | 10 |
| 12.5 | 90 | 10 |
| 13.0 | 35 | 65 |
| 17.0 | 35 | 65 |

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

**MS/MS Parameters (Period 1): MRM transitions are the same for both A and B isomers of 1"-COOH-S-2840**

| Analyte | Transition Ions | Dwell Time | DP | CE | CXP |
|---|---|---|---|---|---|
| **1'-COOH-S-2840** | **362/318** | **200** | **-10** | **-18** | **-5** |
| **1'-COOH-S-2840** | **362/131** | **200** | **-10** | **-35** | **-5** |
| **1'-COOH-S-2840-$d_3$** | **365/121** | **200** | **-10** | **-18** | **-5** |

MRM scan using TurboIonSpray®, Negative Polarity, Unit/Unit Resolution

| CUR | CAD | IS | TEM | GS1 | GS2 | EP |
|---|---|---|---|---|---|---|
| 10 | 8 | -4000 | 500 | 40 | 20 | -10 |

**MS/MS Parameters (Period 2):**

| Analyte | Transition Ions | Dwell Time | DP | CE | CXP |
|---|---|---|---|---|---|
| **3'-OH-S-2840** | **348/175** | **400** | **-10** | **-23** | **-5** |
| **3'-OH-S-2840** | **348/130** | **400** | **-65** | **-35** | **-5** |
| **3'-OH-S-2840-$d_3$** | **351/178** | **400** | **-10** | **-23** | **-5** |

MRM scan using TurboIonSpray®, Negative Polarity, Unit/Unit Resolution

| CUR | CAD | IS | TEM | GS1 | GS2 | EP |
|---|---|---|---|---|---|---|
| 10 | 8 | -4000 | 500 | 40 | 20 | -10 |

**MS/MS Parameters (Period 3):**

| Analyte | Transition Ions | Dwell Time | DP | CE | CXP |
|---|---|---|---|---|---|
| **S-2399** | **334/258** | **400** | **50** | **30** | **19** |
| **S-2399** | **334/238** | **400** | **50** | **45** | **19** |
| **S-2399- $d_3$** | **337/261** | **400** | **50** | **30** | **19** |
| **S-2399-$d_3$** | **337/241** | **400** | **50** | **45** | **19** |

MRM scan using TurboIonSpray®, Positive Polarity, Unit/Unit Resolution

| CUR | CAD | IS | TEM | GS1 | GS2 | EP |
|---|---|---|---|---|---|---|
| 10 | 8 | 4000 | 500 | 40 | 20 | 10 |

Approximate Retention Times:

1'-COOH-S-2840-A: 4.99 minutes
1'-COOH-S-2840-B: 5.30 minutes
3'-OH-S-2840: 7.87 minutes
S-2399: 8.25 minutes

## D. Potential Interferences

1. Matrix Interference

The detection technique is highly selective for this method and no matrix interferences were observed.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. No interferences were observed.

3. Labware Interference

This method uses disposable labware and washable glassware. No interferences from the labware or glassware use were observed.

## E. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS. This method of analysis is highly selective; no additional confirmatory technique was used.

## F. Time Required for Analysis

A period of five hours was required to extract the 15 samples in the validation set and prepare them for analysis on the LC-MS/MS. The LC-MS/MS analysis set was run overnight with approximately 2 hours of data processing the following day for the analytical run.

## G. Modification or Potential Problems

There were no modifications to the method. An equivalent HPLC system was used during the study. Period times for MS/MS acquisition were optimized for the observed analyte retention times.

During the SPE cleanup approximately 3 mL of methanol was lost on the rinse step of the sample bottle of one of the 10X LOQ fortified samples (698ILV01-12). An extra 3 mL of methanol was added to the sample bottle for rinsing, followed by elution of the SPE column in order to compensate for the lost volume. There were no abnormalities observed in the results for this sample.

Initially an equivalent HPLC column with similar dimensions was evaluated prior to the ILV trial. However, equivalent elution of the analytes could not be achieved without modifications to the gradient. An identical column to the one listed in the reference method was purchased and used. Retention times only differed slightly from the reference method and no modifications to the gradient program were necessary. The period change between the elution of 3'-OH-S-2840 and S-2399 is a bit narrow in time. Care needs to be taken to ensure that the period change is timed so that complete acquisition of both peaks can be achieved.

## H. Methods of Calculation

Analyst Chromatography Data System version 1.6, a product of AB Sciex, was used to acquire and integrate the chromatographic peaks. A linear regression with 1/x weighting was generated from the peak area ratio of the calibration standards to the IS. The regression was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted

on the x-axis. Peak area ratio was designated as the dependent variable and plotted on the y-axis.

From this regression, a slope, intercept, a correlation coefficient and other parameters of the standard curve were calculated. The slope and intercept of the weighted regression were used to determine the amount of residues in each sample. Six calibration standard concentrations were injected within the analytical set. Calibration standards were injected every five sample injections as well as at the beginning and end of the injection sequence.

The concentration as µg/L of analyte residue found in samples was calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL \text{ from curve}) \times (\text{Final Vol. (mL)}) \times (1\ \mu g) \times (1000\ mL)}{(\text{Sample Amount (mL)}) \times (1000\ ng) \times (1\ L)}$$

Recovery of each of the analytes from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{Measured\ Concentration\ (\mu g/L)}{Theoretical\ Concentration\ (\mu g/L)}\ X\ 100$$

An example calculation for water, for an S-2399 laboratory fortification sample in set 698ILV01, sample 698ILV01-10 LOQ sample fortified at 1.0 µg/L ppb, is as follows:

*standard curve equation: $y = 1.03(x) + (0.0292)$*

*where x = S-2399 concentration in ng/mL and*

*y = peak response = 74701.3*

*S-2399 concentration from the curve =0.440 ng/mL*

$$\mu g/L = \frac{(0.440\ ng/mL) \times (40\ mL) \times (1\ \mu g) \times (1000\ mL)}{(20\ mL) \times (1000\ ng) \times (1\ L)} = 0.880\ ng/mL$$

$$\%\ Recovery = \frac{0.880\ \mu g/L}{1.00\ \mu g/L}\ X\ 100 = 88.0\%$$

No detectable residues were measured in any control samples. Laboratory fortification samples were not corrected for control responses (no responses were observed). Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

## V. CONCLUSION

The reference method was successfully validated using Liquid Chromatography (LC) equipped with a tandem mass spectrometer (MS/MS) detector. The method was successfully validated at 1.00 and 10.0 ppb for the determination of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in surface water during the first inter-laboratory validation trial.

## VI. PROTOCOL AMENDMENTS/DEVIATIONS

There were two protocol amendments for this study. Protocol amendment 1 made a change to the Sponsor Representative due to structural changes at the Study Sponsor. Protocol amendment 2 made a change in the purity of the 3'-OH-S-2840 reference substance due to the issue of a new Certificate of Analysis. Neither of these amendments had a negative effect on the integrity or results of the study.

There were no protocol deviation for this study.

## VII. CIRCUMSTANCES AFFECTING THE DATA

No circumstances were encountered that would affect the quality or integrity of the data generated in this study.

## VIII. DATA STORAGE AND RECORDS RETENTION

At the conclusion of the study, all original raw data, or certified copies thereof, and summaries of data specific to this study will be archived at GPL. All data will be transferred to the Sponsor after issuance of this report. Original facility records will be maintained at GPL. A copy of this report and analytical raw data will also be maintained at GPL.

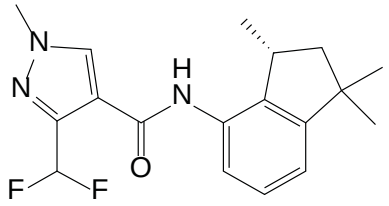
## 1. INTRODUCTION

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).

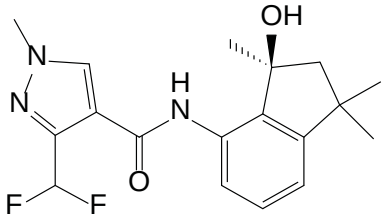
## 2. MATERIALS

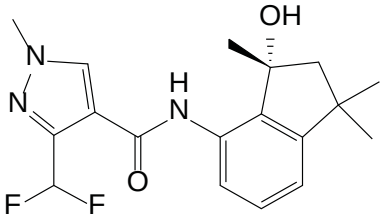
### 2.1 Analytical Reference Standards

The following analytical reference standards are used:

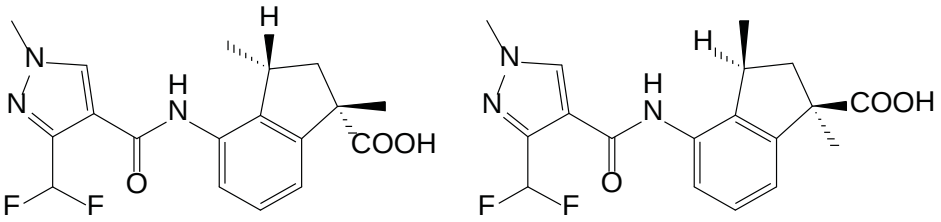



S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3´*R*)-1´,1´,3´-trimethyl-2´,3´-dihydro-1´*H*-inden-4´-yl]-1*H*-pyrazole-4-carboxamide

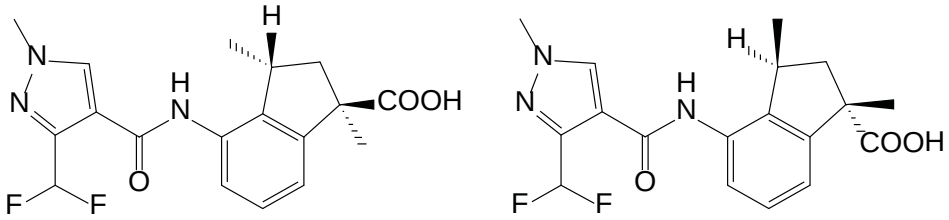



3'-OH-S-2840 (MW = 349.4)
3-(Difluoromethyl)-*N*-[3´-hydroxy-(3´R)-1´,1´,3´-trimethyl-2´,3´-dihydro-1´*H*-inden-4´-yl]-1-methyl-1*H*-pyrazole-4-carboxamide
3-(Difluoromethyl)-*N*-[3´-hydroxy-(3´S)-1´,1´,3´-trimethyl-2´,3´-dihydro-1´*H*-inden-4´-yl]-1-methyl-1*H*-pyrazole-4-carboxamide

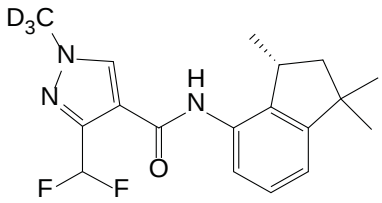


1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

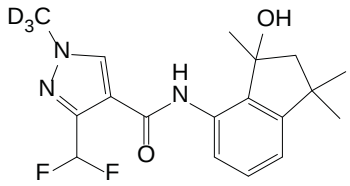


1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

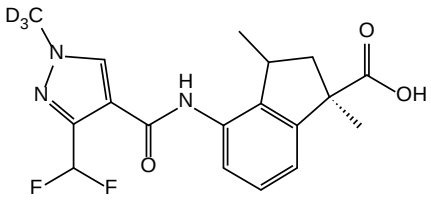
## 2.2 Optional Internal Standards



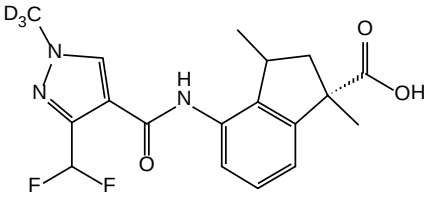
S-2399-$d_3$



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<br>B = methanol |
| Gradient Program: | T = 0 min, 65% A + 35% B<br>T = 1.0 min, 65% A + 35% B<br>T = 6.0 min, 10% A + 90% B<br>T = 7.0 min, 35% A + 65% B<br>T = 10.0 min, 35% A + 65% B<br>T = 10.5 min, 10% A + 90% B<br>T = 12.5 min, 10% A + 90% B<br>T = 13.0 min, 65% A + 35% B<br>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 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 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:

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

*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:

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

*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):

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

## 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.