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 Sediment and Soil" (Method Number RM-50S-1). 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 soil during the second 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 soil sample, 38586-BS-B was supplied by the Sponsor. There was no response in the control matrix samples in the chromatograms corresponding to the retention time of the analytes.

The control soil sample was analyzed using the provided analytical method. Soil samples were extracted twice using a solution of acetone and water via shaking followed by centrifugation. Samples were extracted a third time in a solution of acetone and 0.5 M hydrochloric acid via shaking followed by centrifugation. Soil extracts were buffered first with 0.5 M sodium acetate followed by addition of 1 M acetic acid/1 M sodium acetate solution, centrifuged 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 an internal standard solution, vialed, and analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

The method was validated at 0.01 and 0.1 µg/g in the second attempt for the detection of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in soil.

II. MATERIALS

A. Equipment

The equipment that was used is listed below:

- Analytical balance: Mettler Toledo XS204
- Top Loading balance: Mettler Toledo MS3002S/03
- Reciprocating mechanical shaker, Eberbach
- Volumetric flasks, glass: various sizes
- Bottles, amber glass with Teflon lined cap: various sizes
- Bottles, HDPE: Nalgene 125-mL
- Volumetric glass pipettes: various sizes
- Polypropylene centrifuge tubes: VWR 50-mL
- Graduated cylinders: various volumes
- Graduated mixing cylinders: 100-mL
- 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 HLB, SPE column, 1-g (Cat# 186000117)
- 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
Acetone	VWR	ChromAR®	MK243510
Acetonitrile	Fisher	Optima®	A996
Ammonium Acetate	Fisher	HPLC	A639
Hydrochloric Acid, 12 N	EMD	ACS	HX0603
Methanol	VWR	ChromAR®	MK304110
Sodium Acetate	VWR	ACS	97061-994
Water	Fisher	HPLC	W5-4

Preparation of Reagent Solutions:

0.5 M Sodium Acetate in Water: Prepared by adding 41 g sodium acetate to approximately 800 mL of HPLC-grade water in a 1000-mL mixing cylinder. The sodium acetate was dissolved and the solution was brought up to volume (1000 mL) with HPLC-grade water and mixed well.

0.5 M Hydrochloric Acid Solution: Prepared by adding 41.7 mL of concentrated hydrochloric acid (12 N) to approximately 800 mL of HPLC-grade water into a 1000-mL mixing cylinder and mixed well. The solution was brought up to volume (1000 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.

Acetone/water (4:1, v/v): Prepared by adding 800 mL of acetone and 200 mL of HPLC-grade water and mixing well.

Acetone/0.5 M Hydrochloric acid (4:1, v/v): Prepared by adding 800 mL of acetone and 200 mL of 0.5 M hydrochloric acid solution and mixing well.

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

10 mM Ammonium Acetate in Water: Prepared by adding 0.77 g ammonium acetate to approximately 800 mL of HPLC-grade water. 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 2379b	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-*d3*	AS 2422a	97.2	12/28/2020
3′-OH-S-2840-*d3*	AS 2414a	99.3	11/18/2020
1′-COOH-S-2840-A-*d3*	AS 2420a	99.9	12/21/2020
1′-COOH-S-2840-B-*d3*	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.

Stock solutions of the internal standards were prepared using similar procedures in methanol.

b. Fortification Solutions

High Fortification Solution

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

Low Fortification Solution

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

c. Internal Standard 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 low 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 soil 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 Sediment and Soil" (Method Number RM-50S-1). The limit of quantitation (LOQ) was 0.01 µg/g for all analytes. The method defined limit of detection (LOD) was 0.005 µg/g. A copy of the analytical method is in Appendix B.

The method samples were validated on the second trial as set 687ILV02 on February 16 - 17, 2017. Analytical sets consisted of one reagent blank sample, two control samples, seven LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. 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., 687ILV02-1).

Soil samples were extracted two times using a solution of acetone and water via shaking followed by centrifugation. Samples were extracted a third time in a solution of acetone and 0.5 M hydrochloric acid via shaking followed by centrifugation. Soil extracts were buffered first with 0.5 M sodium acetate followed by addition of 1 M acetic acid/1 M sodium acetate solution, centrifuged 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 an internal standard solution, vialed, and analyzed by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS).

B. Analytical Procedure

1. Control Matrix

The soil control matrix sample, 38586-BS-B, was supplied by the Sponsor and received at GPL on January 31, 2017. The sample was stored frozen when not in use. The soil characterization report was obtained from the sponsor and is presented in Appendix C.

2. Preparation of Samples

Sub-samples (10 g) of the control soil matrix were measured into 50-mL plastic centrifuge tubes.

3. Fortifications

Independent laboratory validation samples were fortified at the LOQ (0.01 µg/g) or 10x the LOQ (0.1 µg/g). Fortifications were completed as follows:

Fortification Level	Amount and Concentration of Spiking Solution Used
LOQ (0.01 µg/g)	100 µL of a 1 µg/mL Low Fortification solution
10x LOQ (0.1 µg/g)	100 µL of a 10 µg/mL High Fortification solution

4. Extraction

After fortification, 25 mL of acetone/water (4:1, v/v) solution was added to each sample and extracted on a platform shaker for 30 minutes at ~200 rpm. Sample extracts were then centrifuged for 5 minutes at 4000 rpm. The sample extracts were decanted into a 125-mL Nalgene bottle. The remaining solid sample in the centrifuge tube was extracted, centrifuged and decanted a second time using the procedures described above. The remaining solid sample was then extracted a third time with 25 mL of acetone/0.5 M hydrochloric acid (4:1, v/v) solution on the platform shaker.

Samples were immediately centrifuged and decanted into their respective 125-mL bottles, thereby combining all three extracts. A 2-mL volume of 0.5 M sodium acetate solution was added to the sample bottles, capped and shaken by hand to mix. A 2-mL volume of 1 M acetic acid/1 M sodium acetate buffer solution was added to the sample bottles, capped and shaken by hand to mix, and then centrifuged.

SPE (Oasis) cartridges were conditioned with 10 mL of methanol followed by equilibration using 10-mL of HPLC-grade water. Gentle vacuum was used as needed for all SPE steps. Entire sample was loaded onto the SPE column in portions into a 50-mL centrifuge tube. SPE extract was transferred to 100-mL mixing cylinder as volume dictated. The sample bottle was then rinsed and the cartridge washed with four 5-mL volumes of methanol and collected into the 50-mL centrifuge tube, followed by transfer to the 100-mL mixing cylinder. Extract volume in the mixing cylinder was brought up to 100 mL with methanol.

An aliquot of 0.5 mL of sample was combined with 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) 10 mM Ammonium Acetate in Water
Flow Rate:	0.7 mL/minute
Run Time:	19.0 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
14.5	90	10
15.0	35	65
19.0	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-*d3*	**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-*d3*	**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-*d3*	**337/261**	**400**	**50**	**30**	**19**
S-2399-*d3*	**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: 5.13 minutes
1'-COOH-S-2840-B: 5.45 minutes
3'-OH-S-2840: 7.95 minutes
S-2399: 8.31 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 eight 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. Modifications or Potential Problems

Two modifications were made to the method in 687ILV02. The first modification was adding another centrifuge step following the addition of the 2 mL of 1 M acetic acid/sodium acetate buffer and shaking by hand. The centrifuge step was included because additional precipitation of sample material was observed following addition of the buffer. The precipitate could cause plugging of the SPE cartridge in the subsequent decanting and loading steps. The second modification included rinsing the Nalgene bottle with 5 mL of methanol on to the SPE cartridge loading step and repeating three more times instead of repeating one time as described in

the reference method. This modification was necessary to achieve acceptable recoveries of the analytes as described in section **IV. RESULTS/DISCUSSION**.

An equivalent HPLC system was used for the study. Period times for MS/MS acquisition were optimized for the observed analyte retention times. 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/g of analyte residue found in samples was calculated with Microsoft® Excel using the following equation:

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

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

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

An example calculation for soil, for an S-2399 laboratory fortification sample in set 687ILV02, sample 687ILV02-10 LOQ sample fortified at 0.01 µg/g, is as follows:

standard curve equation: $y = 1.04(x) + (0.00524)$

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

y = peak ratio response = 154386.0/338110.7

S-2399 concentration from the curve =0.432 ng/mL

$$\mu g/g = \frac{(0.432\ ng/mL) \times (200\ mL) \times 1\ \mu g}{(10.00\ g) \times 1000\ ng} = 0.00864\ \mu g/g$$

$$\%\ Recovery = \frac{0.00864\ \mu g/g}{0.0100\ \mu g/g} \times 100 = 86.4\%$$

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.

I. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), coefficient of variation, and 95% confidence interval for analyte recovery data. Linear regression analysis using weighting as defined in the method (see section H above) was applied to generate LC-MS/MS calibration curves for the determination of slope, y-intercept and correlation coefficient values.

The method is specific for S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B due to the use of liquid chromatography coupled with tandem mass spectrometric detection including detection of two analyte transition ion pairs.

G. Limitations

The method has been independently validated in soil obtained from a specific source. It can be assumed that the method is applicable to other soil types not tested in this validation provided successful recovery tests are conducted at relevant fortification levels.

The SPE elution step with methanol may not be complete at the volume given in the reference method. The total methanol elution volume was doubled in trial 2, as described in section **III. G. Modifications or Potential Problems**, in order to improve recoveries of the analytes.

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.

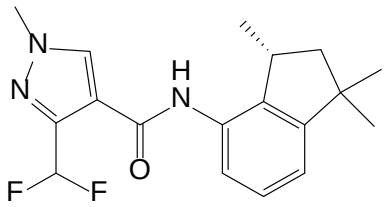
1. INTRODUCTION

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 sediment and soil. Each S-2840 metabolite includes enantiomers. For the carboxylic acid metabolites, the A and B designations of the acids are based on their isomeric similarities. 1'-COOH-S-2840-A has two enantiomers and 1'-COOH-S-2840-B also contains two enantiomers; however, 1'-COOH-S-2840-A and 1'-COOH-S-2840-B are diastereomers. This method is a modification of Valent method RM-50S. Briefly, the residues are extracted from sediment or soil using two extractions of acetone/water (4/1, v/v) followed by an extraction with acetone/0.5M HCl (4/1, v/v). The combined extract is adjusted to pH ca. = 5 and undergoes solid phase extraction (SPE) utilizing an Oasis HLB 20cc (1 g) cartridge. Analytes are eluted and sample eluent total volumes are adjusted to 100 mL using methanol. Samples are reconstituted in 1:1 (v/v) methanol: water (with or without internal standard) and analyzed using high-performance liquid chromatography with tandem mass spectrometry LC/MS-MS (with turbo-ion spray ionization in positive and negative ion modes).

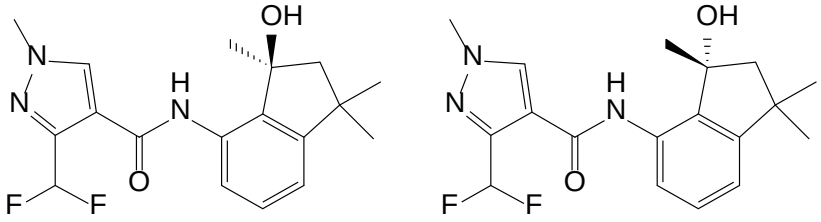
2. MATERIALS

2.1 Analytical Reference Standards

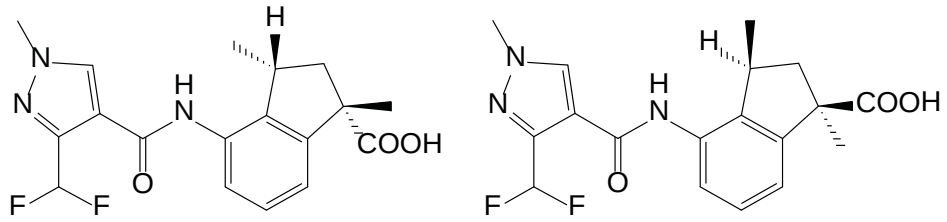
The following analytical reference standards are used:



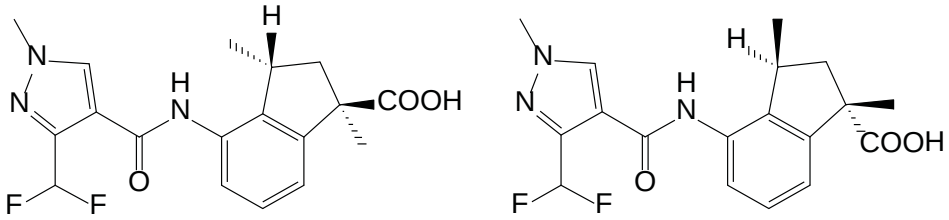
S-2399 (MW = 333.4)
3-(Difluoromethyl)-1-methyl-*N*-[(3´*R*)-1´,1´,3´-trimethyl-2´,3´-dihydro-1´*H*-inden-4´-yl]-1*H*-pyrazole-4-carboxamide



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

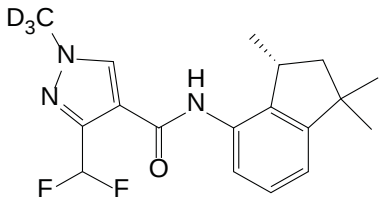


1'-COOH-S-2840-A (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

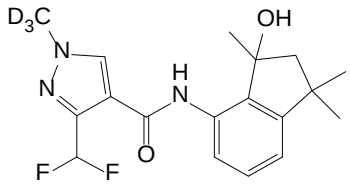


1'-COOH-S-2840-B (MW = 363.4)
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′R,3′R)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic acid
4′-({[3-(Difluoromethyl)-1-methyl-1*H*-pyrazol-4-yl]carbonyl}amino)-(1′S,3′S)-1′,3′-dimethyl-2′,3′ dihydro-1′*H*-indene-1′-carboxylic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

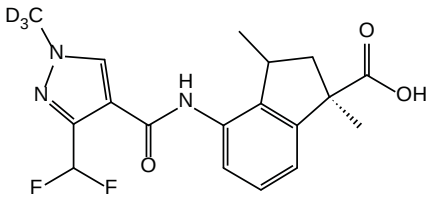
2.2 Optional Internal Standards



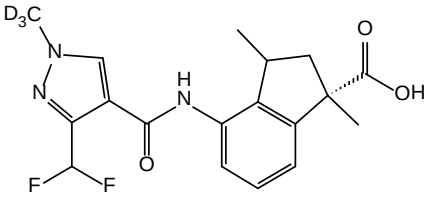
S-2399-d_3



3'-OH-S-2840-d_3



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



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

2.3 Analytical Reference Standard Preparation

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

Stock Solutions, 1 mg/mL:

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

Intermediate Solution, 10 µg/mL:

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

Fortification Solution, 1 µg/mL:

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

Calibration Standard Solutions:

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

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

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

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

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

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

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

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

2.3.1 Optional Internal Standard Preparation

Internal Stock Solutions, 1 mg/mL:

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

Intermediate Internal Standard Solution, 1 µg/mL:

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

Internal Standard Final Volume Solution, 2 µg/L:

Transfer a 2.0 mL aliquot of the 1 µg/mL Intermediate Internal Standard Solution to a 1000 mL volumetric flask, and dilute to volume with methanol/HPLC water (1/1, v/v). Store this solution in a refrigerator or freezer when not in use.

Internal Standard Final Volume Solution, 1 µg/L:

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

2.4 Reagents

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

2.5 Reagent Solution Preparation

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

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

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

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

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

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

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

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

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

3 EQUIPMENT

Autosampler vials, screw-top with Teflon-coated septa
Balances, analytical and top-loading
Centrifuge
Centrifuge tubes, polypropylene, 50 mL graduated with caps (BD Falcon #2098 or equivalent)

Freezer, -20°C capable
Glass bottles, 4 oz amber with Teflon-coated caps (VWR Cat. No. 36319-435 or equivalent)
Glass vials (approximately 22 mL or equivalent)
Graduated cylinder, stoppered (100 mL)
High-performance Liquid Chromatograph (Agilent Technologies 1200 series or equivalent)
Mass Spectrometer (Applied Biosystems API 4000 or equivalent)
Pipette(s), Pasteur, volumetric and/or automatic (volumes of 0.20 to 20 mL)
Reciprocating mechanical shaker, (Erbach or equivalent)
Refrigerator
Solid phase extraction cartridge (Oasis HLB, 1g Waters # 186000117 or equivalent)
Storage containers, polypropylene, 125-mL with caps (Cole Parmer # 06041-12)
Volumetric flasks, (**pre-rinsed with methanol**) assorted volumes as needed

4 INSTRUMENTATION

High Performance Liquid Chromatograph with Mass Spectrometry (LC/MS-MS) – Agilent Technologies 1200 series HPLC with tandem Applied Biosystems API 4000 mass selective detector, with turbo ion spray ionization in positive and negative ion modes. Conditions shown below are suggested for this analysis. The conditions may be modified as needed to optimize the chromatography, to resolve matrix interferences, or to utilize other types of LC/MS-MS instruments. The LC/MS-MS parameters that are used must be documented with each chromatographic set.

HPLC Conditions:

Column: Eclipse XDB-C8, 5µm, 150 mm x 4.6mm, Agilent part # 993967-906	
Column Oven Temperature:	40 ± 1°C
Mobile Phase:	A = 10mM ammonium acetate in HPLC water B = methanol
Gradient Program:	T = 0 min, 65% A + 35% B T = 1.0 min, 65% A + 35% B T = 6.0 min, 10% A + 90% B T = 7.0 min, 35% A + 65% B T = 10.0 min, 35% A + 65% B T = 10.5 min, 10% A + 90% B T = 14.5 min, 10% A + 90% B T = 15.0 min, 65% A + 35% B T = 19.0 min, 65% A + 35% B
Flow Rate Program:	700 µL/min
Injection Volume:	25 µL

Typical MS-MS Parameters:

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

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi (N_2)
Curtain gas (CUR):	10 psi (N_2)
Gas sources:	GS1 = 20 psi (N_2), GS2: 20 psi (N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
1'-COOH-S-2840-A	362	318, (131)*	200	-10	-10	-18, (-30)	-5
1'-COOH-S-2840-A- *d3*	365	321	200	-10	-10	-18	-5
1'-COOH-S-2840-B	362	318, (131)	200	-10	-10	-18, (-30)	-5
1'-COOH-S-2840-B- *d3*	365	321	200	-10	-10	-18	-5

*Values in parentheses are for qualifier / confirmatory ions.

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

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi (N_2)
Curtain gas (CUR):	10 psi (N_2)
Gas sources: GS1 =	20 psi (N_2), GS2: 20 psi (N_2)
Ion spray voltage (IS):	-4000 V
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
3'-OH-S-2840	348	175, (130)*	400	-10, (-65)	-10	-23 (-35)	-5 (-10)
3'-OH-S-2840- *d3*	351	178	400	-10	-10	-23	-5

*Values in parentheses are for qualifier / confirmatory ions.

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

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	8 psi (N_2)
Curtain gas (CUR):	10 psi (N_2)
Gas sources: GS1 =	20 psi (N_2), GS2: 20 psi (N_2)
Ion spray voltage (IS):	4000 V
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (amu)	Product ion Q3 (amu)	Scan time (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
S-2399	334	238, (258)*	400	55	10	45, (27)	19
S-2399- *d3*	337	241, (261)	400	55	10	45, (27)	19

*Values in parentheses are for qualifier / confirmatory ions.

5 ANALYTICAL PROCEDURES

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

1. Sample Preparation

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

   Weigh 10.0 g (± 0.1 g) of the homogenized sample into a 50 mL polypropylene centrifuge tube. At this point, if required by the testing facility, control samples for method recovery should be fortified with S-2399, 3'-OH-S-2840, 1'-COOH-S-2840-A, and 1'-COOH-S-2840-B (See Section 11 NOTES).

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

2. Sample Extraction

   Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes at approximately 4000 rpm or as needed to separate the solids from the extraction solvent. Decant the sample extract into a 125-mL polypropylene (or other suitable) container.

Add 25 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample. Disintegrate the pellet, if needed and shake on reciprocating shaker for 30 minutes. Centrifuge the sample for approximately 5 minutes and decant the sample extract into the 125-mL polypropylene (or other suitable) container containing the first extract.

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

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

3. pH Adjustment

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

4. SPE Cartridge Conditioning, Loading and Extraction

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

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

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

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

5. Final Analyte Solution Preparation and Injection

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

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

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

6. LC/MS-MS ANALYSIS

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

For a linear calibration or 2^{nd}-order polynomial calibration, analyze a minimum of five calibration standard concentrations within the analytical sequence. A typical set of standards includes concentrations of 0.50, 1, 2.5, 5, 10 and the required 0.25 µg/L standard (with an injection volume of 25 µL).

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

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

If the detector response observed for a sample is greater than the detector response of the highest calibration standard, the sample extract must be diluted and the diluted extract analyzed. The sample extract must be diluted such that the detector response obtained is within the calibrated response range of the LC/MS-MS.

7 CALCULATIONS

To calculate the line or curve for instrument calibration, the peak area (or peak area ratio) and the concentration of each of the calibration standards are input into an Excel spreadsheet. The data are fit to a linear regression (weighted relative to 1/concentration). The inputs are based on the standard concentration and the observed analyte peak area ([or peak area ratio] or expressed as Peak Units; e.g., as area/10^6). Replicate entries are included in the data set prior to performing

the regression in Excel (to provide weighting relative to 1/concentration).

For example:

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

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

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

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

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

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

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

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

peak units in the control sample are equal to or greater than the lowest calibration standard, then the concentration observed in the control sample is subtracted from the concentration observed in the fortified sample to provide a corrected concentration. Otherwise, the peak units in the control sample are subtracted from the peak units in the fortified sample prior to calculating a corrected concentration. This corrected concentration is then used to calculate percent recovery:

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

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

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

8 LIMIT OF DETECTION

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

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

9 LIMIT OF QUANTIFICATION

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

10 CHROMATOGRAMS

Example chromatograms are shown in Figures 1 through 16.

11 NOTES

Fortified control samples are to be analyzed with each set of samples. Method recoveries must be 70 to 120% to be acceptable, unless approved by the supervising chemist responsible for the analysis, or by the Study Director. If the testing facility does not require concurrent analysis of fortified control samples, or if a UTC sample is not available, this method requirement may be waived.