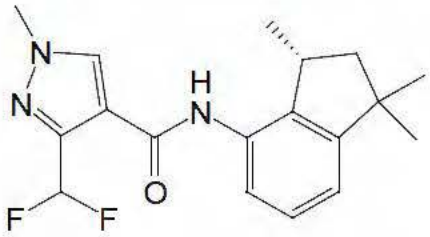
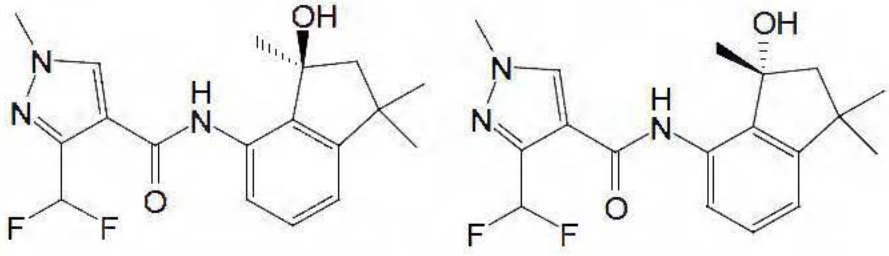
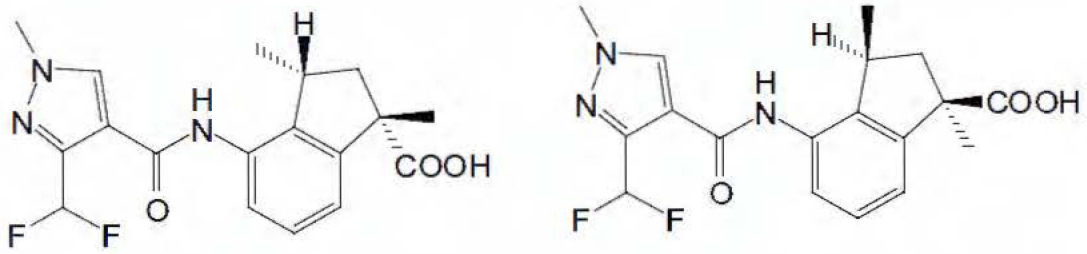
Analytical Reference Standards

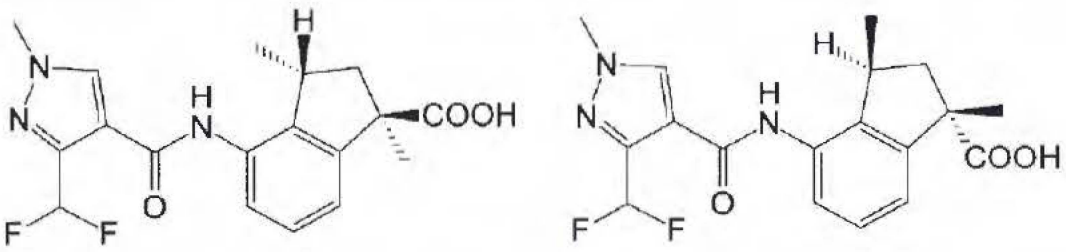
Standard name:	S-2399
Lot number:	AS 2375a
CPS ID:	15-CPS-Oct06-05
Valent sample archive no.:	V-Arc-2376
Source:	Valent U.S.A.
Purity:	95.3%
Date of analysis:	05 Oct 2015
Expiration date:	05 Oct 2016
Storage conditions:	Freezer
Molecular weight:	333.4
Molecular structure:	

Standard name:	3′-OH-S-2840
Lot number:	AS 2379b
CPS ID:	15-CPS-Oct06-04
Valent sample archive no.:	V-Arc-2489
Source:	Valent U.S.A.
Purity:	97.8%
Date of analysis:	21 Sep 2015
Expiration date:	21 Sep 2016
Storage conditions:	Freezer
Molecular weight:	349.4
Molecular structure:	

Standard name:	1′-COOH-S-2840A
Lot number:	AS 2393a
CPS ID:	15-CPS-Oct06-02
Valent sample archive no.:	V-Arc-2437
Source:	Valent U.S.A.
Purity:	100%
Date of analysis:	12 Feb 2015
Expiration date:	12 Feb 2016
Storage conditions:	Freezer
Molecular weight:	363.4
Molecular structure:	



Standard name:	1′-COOH-S-2840B
Lot number:	AS 2394a
CPS ID:	15-CPS-Oct06-03
Valent sample archive no.:	V-Arc-2438
Source:	Valent U.S.A.
Purity:	99.6%
Date of analysis:	12 Feb 2015
Expiration date:	12 Feb 2016
Storage conditions:	Freezer
Molecular weight:	363.4
Molecular structure:	



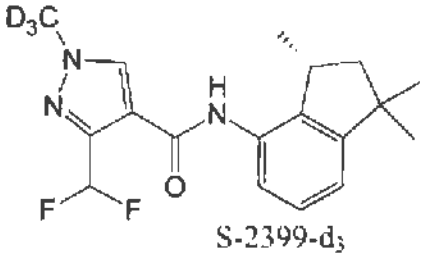
Internal Standards

20 mL of a methanol solution (Valent ID # RS-50-42-1 WS) containing a mixture of each of the following internal standards at a concentration of 1 µg/mL was provided by Valent U.S.A. This intermediate internal standard solution was prepared by Valent on 13 Jul 2015 with an expiration date of 13 Jul 2017. The solution was assigned CPS ID # 15-CPS-Sep11-01 and stored refrigerated when not in use.

Standard name: S-2399-d_3

Molecular weight: 336.4

Molecular structure:

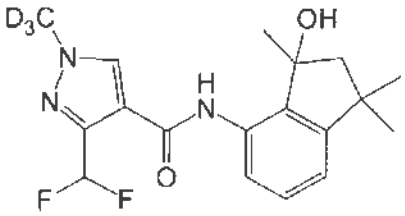


S-2399-d_3

Standard name: 3′-OH-S-2840-d_3

Molecular weight: 352.4

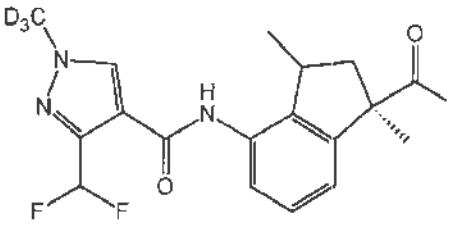
Molecular structure:



Standard name: 1′-COOH-S-2840A-d_3

Molecular weight: 366.4

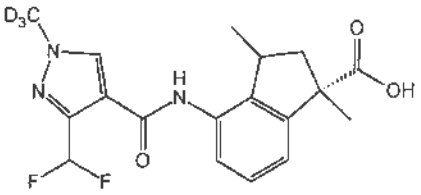
Molecular structure:



Standard name: 1′-COOH-S-2840B-d_3

Molecular weight: 366.4

Molecular structure:



2.0 INTRODUCTION

The objective of this study was to validate Valent method RM-50S "Determination of Residues of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in Soil" [1]. This method passed the ILV for both analytes in soil on the first attempt with no major modifications.

This study was designed to fulfill the requirements of the US EPA Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2]. In addition, this study was conducted in compliance with US EPA FIFRA (40 CFR Part 160) GLP standards [3].

3.0 MATERIALS AND METHODS

3.1 Test Substances

Standard name: S-2399
Lot: AS 2375a
CPS ID: 15-CPS-Oct06-05
Sample archive no.: V-Arc-2376
Purity: 95.3%
Date of analysis: 05 Oct 2015
Expiration date: 05 Oct 2016
Storage conditions: Freezer

Standard name: 3′-OH-S-2840
Lot: AS 2379b
CPS ID: 15-CPS-Oct06-04
Sample archive no.: V-Arc-2489
Purity: 97.8%
Date of analysis: 21 Sep 2015
Expiration date: 21 Sep 2016
Storage conditions: Freezer

Standard name: 1′-COOH-S-2840A
Lot: AS 2393a
CPS ID: 15-CPS-Oct06-02
Sample archive no.: V-Arc-2437
Purity: 100%
Date of analysis: 12 Feb 2015
Expiration date: 12 Feb 2016
Storage conditions: Freezer

Standard name: 1′-COOH-S-2840B
Lot: AS 2394a
CPS ID: 15-CPS-Oct06-03
Sample archive no.: V-Arc-2438
Purity: 99.6%
Date of analysis: 12 Feb 2015
Expiration date: 12 Feb 2016
Storage conditions: Freezer

3.2 Test System

The test system used for the validation was a control soil sample collected at a site in Washington, USA and provided by Valent. See Appendix 1 for soil characterization report. The sample was assigned CPS ID # GS-15-60-1 and stored in a sealed container under ambient conditions when not in use.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in pages 5 through 7 of the Valent method (RM-50S; included in the protocol in Appendix 2) and precisely documented in the study records (CPS Notebook 0221, pages 04–07 c.). Identical or equivalent equipment and materials were used.

3.3.1 Equipment and Apparatus

Analytical balance (Mettler Toledo)
Volumetric pipettes, assorted volumes
Electronic and manual pipettors of multiple volumes
Refrigerator/Freezer (Nor-Lake® Scientific)
Refrigerator (SciCool)
Top-loading balance (A&D Company)
Allegra® X-22R centrifuge (Beckman Coulter, Inc.)
Ultrasonic cleaner 5210 (Branson)
Millipore Direct– Q5™ Water Purification System
Reciprocating shaker (IKA Labortechnik)
Funnels, 65-mm
Glass wool
50-mL polypropylene centrifuge tubes
Pasteur pipettes, various sizes
Separatory funnels 250 mL
N-Evap with water bath and thermometer (Organomation Associates, Inc.)
Vortex (VWR International)
Various sizes of volumetric flasks
Glass graduated cylinders, 100-mL with stoppers
Agilent Zorbax Eclipse XDB-C8 HPLC Column, 4.6 × 150 mm, 5 μm

LC-MS/MS - Agilent 1200 binary pump HPLC system and autosampler, coupled to an Applied Biosystems® API 4000™ mass spectrometer with an electrospray ionization interface.

3.3.2 Reagents

Acetone (BDH), HPLC grade
Milli-Q water
Methanol (EMD-Millipore), LC-MS grade
Hydrochloric acid, 12 N (EMD-Millipore), ACS grade
Dichloromethane (EMD-Millipore), OmniSolv
Ammonium acetate, (Fluka/Sigma-Aldrich), HPLC grade
Sodium acetate, trihydrate (Sigma-Aldrich), ACS grade
Sodium chloride, (Sigma-Aldrich), reagent grade
Sodium sulfate, anhydrous (EMD-Millipore), ACS grade

3.3.3 Reagent Solution Preparation

Acetone/Water (4:1, v/v)
Added 800 mL of acetone and 200 mL of Milli-Q water sequentially into a reagent bottle and mixed well.

Methanol/Water (1:1, v/v)
Added 1000 mL of methanol and 1000 mL of Milli-Q water sequentially into a reagent bottle and mixed well.

0.5 M HCL Solution
Diluted 4.17 mL of 12N HCL into 100 mL of Milli-Q water.

Acetone/0.5 M HCL (4:1, v/v)
Added 400 mL of acetone and 100 mL of 0.5 M HCL solution sequentially into a reagent bottle and mixed well.

0.5 M Sodium Acetate Solution
Dissolved 34.0 g of sodium acetate trihydrate in 500 mL of Milli-Q water.

Sodium Chloride/Water (5%, w/v)
Dissolved 50.0 g of sodium chloride in 1000 mL of deionized water.

5 mM Ammonium Acetate in Water (mobile phase A)
Dissolved 0.385 g of ammonium acetate in 1000 mL of Milli-Q water.

5 mM Ammonium Acetate in Methanol (mobile phase B)
Dissolved 0.385 g of ammonium acetate in 1000 mL of methanol.

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument sensitivity, and linearity of instrument responses to a range of analyte concentrations were determined to demonstrate that the method was under control.

3.4.2 Standard Solutions Preparation

The internal standard final volume solution (IS-1, 1 μg/L) for each analyte (S-2399-d_3, 3′-OH-S-2840-d_3, 1′-COOH-S-2840A-d_3, and 1′-COOH-S-2840B-d_3) was prepared by transferring a 1.00 mL aliquot of the 1 μg/mL intermediate internal standard solution (Valent ID # RS-50-42-1 WS, CPS ID # 15-CPS-Sep11-01) into a 1000-mL volumetric flask and diluting to volume with methanol/water (1/1, v/v). This solution was stored refrigerated when not in use.

The primary stock solution (approximately 1.00 mg/mL) for each analyte (S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B) was prepared separately by weighing approximately 10.0 mg (amount corrected for chemical purity) of compound onto a tared piece of weigh paper and transferring into a 10-mL glass volumetric flask. Each pre-weighed standard was dissolved and diluted to volume with acetone. The stock solutions were stored in the freezer when not in use.

Fortification solution FS-1, containing 10.0 μg/mL of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B was prepared by transferring an appropriate volume (approximately 1000 μL) of each of the primary stock solutions into a 100-mL volumetric flask and diluting to volume with acetone. A second fortification solution, FS-2, was prepared at a concentration of 1.00 μg/mL by measuring 10.0 mL of FS-1 into a 100-mL volumetric flask and diluting to volume with acetone. The fortification solutions were stored in freezer when not in use.

The calibration standard solutions ranged from 0.250 μg/L to 10.0 μg/L for each analyte and were prepared in the following manner:

CS-1 (10.0 μg/L): A 0.500 mL aliquot of the 1.00 μg/mL fortification solution FS-2 was transferred into a 50.0-mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

CS-2 (5.00 μg/L): A 25.0 mL aliquot of the 10.0 μg/L calibration standard solution CS-1 was transferred into a 50.0 mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

CS-3 (2.50 μg/L): A 25.0 mL aliquot of the 5.00 μg/L calibration standard solution CS-2 was transferred into a 50.0 mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

CS-4 (1.00 μg/L): A 20.0 mL aliquot of the 2.50 μg/L calibration standard solution CS-3 was transferred into a 50.0 mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

CS-5 (0.500 μg/L): A 25.0 mL aliquot of the 1.00 μg/L calibration standard solution CS-4 was transferred into a 50.0 mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

CS-6 (0.250 μg/L): A 25.0 mL aliquot of the 0.500 μg/L calibration standard solution CS-5 was transferred into a 50.0 mL volumetric flask and diluted to volume with the internal standard final volume solution IS-1.

The final internal standard concentration is 1 μg/L for all calibration standards. Calibration standard solutions were stored in freezer when not in use.

3.4.3 Sample Validation Sets, Fortification, and Extraction Procedure

Sample Validation Sets

The analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B at the limit of quantitation (LOQ, 0.0100 ppm), and five untreated controls fortified with S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B at 10× LOQ (0.100 ppm). Fortifications will be made by addition of a fortification standard into the control matrix via pipette.

Sample Preparation for Analysis

1. Weighed 10.0 ± 0.1 g of soil into a 50-mL polypropylene centrifuge tube.
2. Added the appropriate amount of fortification solution to the sample.
    a. For the reagent blank and control samples, added nothing.
    b. For the LOQ samples, added 100 μL of FS-2 (1.00 μg/mL of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B) fortification solution onto the soil.
    c. For the 10× LOQ samples, added 100 μL of FS-1 (10.0 μg/mL of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B) fortification solution onto the soil.
3. Added 25.0 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shook on reciprocating shaker for 30 minutes.
4. Centrifuged the sample for 5 minutes at 2000 rpm to separate the solids from the extraction solvent and decanted the extract into a stoppered 100-mL mixing graduated cylinder.
5. Added 25.0 mL of acetone/water (4/1, v/v) to the centrifuge tube containing the sample and shook on reciprocating shaker for 30 minutes.

6. Centrifuged the sample for 5 minutes at 2000 rpm to separate the solids from the extraction solvent and decanted the extract into the 100-mL graduated cylinder containing the first extract.
7. Added 25.0 mL of acetone/0.5 M HCl (4/1, v/v) to the centrifuge tube containing the sample and shook on reciprocating shaker for 30 minutes.
8. Immediately centrifuged the sample for 5 minutes at 2000 rpm to separate the solids from the extraction solvent and decanted the extract into the 100-mL graduated cylinder containing the first two extracts.
9. Immediately added 2.00 mL 0.5 M sodium acetate solution to the graduated cylinder and mixed.
10. Diluted extract in graduated cylinder to 100 mL with acetone/water (4/1, v/v) and mixed. Following removal of an aliquot for the following sodium chloride/dichloromethane partition, the extract was stored refrigerated in the stoppered 100-mL mixing graduated cylinder.
11. Added 10.0 mL of 5% sodium chloride solution and 20.0 mL dichloromethane sequentially to a 250-mL separatory funnel.
12. Remixed the contents of the graduated cylinder just prior to adding a 1.00 mL aliquot of the extract to the 250-mL separatory funnel.
13. Immediately shook the separatory funnel for 1 minute and allowed the contents to separate.
14. Drained the lower dichloromethane layer through a funnel containing 20.0 ± 0.1 g anhydrous sodium sulfate suspended on a plug of glass wool into a 50-mL polypropylene centrifuge tube.
15. Added an additional 20.0 mL of dichloromethane to the separatory funnel and shook the separatory funnel for 1 minute and allowed the contents to separate.
16. Drained the lower dichloromethane layer through the funnel containing sodium sulfate into the 50-mL centrifuge tube containing the first extract.
17. Evaporated dichloromethane extract contained in the tube to dryness using an N-Evap with water bath set to <40°C.
18. Re-dissolved (sonicate and vortex) the extract in 2.00 mL of internal standard final volume solution (IS-1, 1 μg/mL).
19. Transferred a portion of the final volume extract to a 2-mL HPLC autosampler vial for analysis. Any remaining final volume extract was stored in a freezer.

3.4.4 Sample Analysis

The samples were analyzed as described by the Valent method RM-50S. After conditioning the instrument with at least 5 injections of a sample extract, the samples were analyzed with six calibration standards and diluent blanks (methanol/water, 1/1, v/v) interspersed with the samples in a sequence. The continuing calibration standard (CS-3, 2.50 μg/L) was injected at the beginning, middle, and end of the sequence.

3.5 LC-MS/MS Instrumentation

Agilent 1200 HPLC System (Agilent Technologies)
API 4000™ Tandem Mass Spectrometer, MS/MS (Applied Biosystems®)
HPLC Column: Agilent Zorbax Eclipse XDB-C8 HPLC Column, 4.6 × 150 mm, 5 μm
Software: Applied Biosystems® Analyst® 1.6.2
Refer to Table 3 for details of the instrument conditions.

3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst® software version 1.6.2. The concentrations of the analytes (S-2399, 3′-0H-S-2840, 1′-COOH-S-2840A, and 1′- COOH-S-2840B) in each sample extract were calculated using a second-order polynomial equation on the basis of the MS detector peak area ratio (analyte peak area response/internal standard peak area response). The data were presented graphically as concentration of the calibration standards versus their peak area ratios using an Excel® spreadsheet to determine the curve parameters and calculate the sample residues. The peak area ratios were entered as the *x* values and concentrations as the *y* values to calculate a curve expressed as the following equation:

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

The data were weighted relative to the concentration of the highest standard (the largest calibration standard concentration was divided by a calibration standard concentration to get the number of entries in a data set). The calibration standard set, with the number of data entries is shown below.

Standard Concentration	Number of Entries in Data Set
10.0 μg/L	1
5.00 μg/L	2
2.50 μg/L	4
1.00 μg/L	10
0.500 μg/L	20
0.250 μg/L	40

Recovery results of each analyte were calculated by multi-point calibration for each sample. The equations used for quantification and an example calculation are presented in Appendix 3. A statistical treatment of the data includes the calculation of means, standard deviations (SD), RSDs as percentages (%), and the 95% confidence intervals. All statistics were calculated using Microsoft® Office Excel 2010.

The limit of quantitation (LOQ) of this method is 0.01 mg/kg (ppm), based on the lowest fortification level validated in this method [1].

The limit of detection (LOD) of this method is 0.005 mg/kg (ppm) [1].

4.3 Potential Interferences and Recommendations

Interference was observed in two samples for the analysis of 1′-COOH-S-2840B. One untreated control and one sample at the LOQ fortification level exhibited higher than expected 1′-COOH-S-2840B concentrations. This caused recovery results to be out of specification for this analyte at the LOQ fortification level. Subsequently, a re-partition of the original extracts (one reagent blank, two untreated controls, and five control samples fortified

at LOQ) was performed. Upon analysis, the re-partitioned samples did not exhibit any interference and the 1′-COOH-S-2840B met specifications. This demonstrated that the interference was introduced in the laboratory during, or after, the sodium chloride/dichloromethane partition of the original sample preparation.

In Section 2.6 of the analytical method Reagent Solution Preparation it is recommended to add Methanol/Water (1:1, v/v) preparation.

4.4 Time Required for Analysis

It took approximately 8 man hours to complete the extraction of one set of 13 samples (one reagent blank, two unfortified matrix control samples, and 10 fortified samples). Time of analysis was approximately 6.5 hours. Completing one set, including extraction and analysis, thus took approximately 1.5 days.

4.5 Communication with Sponsor

Throughout the study, e-mails were exchanged between the study director and sponsor representative regarding study progress.

During method establishment, the study director requested and received clarification of the following from the study monitor:

- Maximum storage time for the standard and final sample solutions.
- The use of separate period for the MS-MS analysis in negative mode.
- The need to quantify a confirmatory transition for each analyte.

Following the first trial, the study director reported that interference was observed in two samples (an untreated control sample and one LOQ fortification sample) causing out of specification recovery results for analyte 1′-COOH-S-2840B at the LOQ fortification level. Study director requested to re-partition extracts of the reagent blank, two untreated controls, and five control samples fortified at LOQ. The sponsor representative agreed. Upon analysis, the re-partitioned samples did not exhibit any interference and the 1′-COOH-S-2840B met specifications. Results were reported and accepted by the sponsor representative.

The independent laboratory method validation was successfully completed without technical communication with the sponsor representative that would have resulted in additions or modifications to the original method. A complete record of all correspondence between the study director and sponsor representative can be found in the study data package.

5.0 CONCLUSIONS

CPS successfully completed an independent validation of Valent analytical method RM-50S “Determination of Residues of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH-S-2840-B in Soil”. The Valent analytical method RM-50S is suitable for determining the residues of S-2399, 3′-OH-S-2840, 1′-COOH-S-2840A, and 1′-COOH-S-2840B in soil down to a level of 0.01 ppm.

Table 3 LC-MS/MS System Operating Parameters

HPLC system:	Agilent Model 1200
MS/MS system:	Applied Biosystems API 4000 mass spectrometer with an electrospray ionization interface
Software:	Applied Biosystems® Analyst® 1.6.2
HPLC column:	Zorbax Eclipse XDB-C8, 4.6 × 150 mm, 5 μm, s/n USRK076260
Column oven temp.:	40 ± 1°C
Mobile phase:	(A—Aqueous): 5 mM Ammonium acetate in Milli-Q water (B—Organic): 5 mM Ammonium acetate in methanol
Injection volume:	25.0 μL
Draw speed:	200.0 μL/min.
Eject speed:	200.0 μL/min.
Needle wash:	Water/methanol (50/50, v/v)
Wash time:	Flush port, 7 seconds
Run Time:	16 minutes

Gradient:

Time (min.)	Flow (μL/min.)	A (%)	B (%)
0.00	700	65.0	35.0
1.00	700	65.0	35.0
6.00	700	10.0	90.0
7.00	700	35.0	65.0
10.00	700	35.0	65.0
11.00	700	65.0	35.0
16.00	700	65.0	35.0

Period 1

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	7 psi (N_2)
Curtain gas (CUR):	15 psi (N_2)
Gas source GS-1:	25 psi (N_2)
Gas source GS-2:	20 psi (N_2)
Ion spray voltage (IS):	-2500 v
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (Da)	Product ion Q3 (Da)	Scan time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
1′-COOH-S-2840A	362.0	318.0, (130.9)	200	-50	-10	-18, (-36)	-19
1′-COOH-S-2840A-d_3	365.0	321.0	200	-40	-10	-16	-17
1′-COOH-S-2840B	362.0	318.0, (130.9)	200	-50	-10	-18, (-36)	-19
1′-COOH-S-2840B-d_3	365.0	321.0	200	-40	-10	-16	-17

( ) = confirmatory ion

Period 2

Scan Type:	MRM
Mode:	Negative
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	7 psi (N_2)
Curtain gas (CUR):	15 psi (N_2)
Gas source GS-1:	25 psi (N_2)
Gas source GS-2:	20 psi (N_2)
Ion spray voltage (IS):	-2500 v
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (Da)	Product ion Q3 (Da)	Scan time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
3′-OH-S-2840	348.0	174.9, (130.9)	200	-10	-10	-44, (-58)	-11, (-13)
3′-OH-S-2840-d_3	351.0	177.9	200	-10	-10	-44	-11

( ) = confirmatory ion

Period 3

Scan Type:	MRM
Mode:	Positive
Ion source:	Turbo V™
Probe Type:	Electrospray
Collision gas (CAD):	7 psi (N_2)
Curtain gas (CUR):	15 psi (N_2)
Gas source GS-1:	25 psi (N_2)
Gas source GS-2:	20 psi (N_2)
Ion spray voltage (IS):	4000 v
Temperature (TEM):	500°C
Interface heater (IH):	On

Analyte	Precursor ion Q1 (Da)	Product ion Q3 (Da)	Scan time (msec)	DP (V)	EP (V)	CE (V)	CXP (V)
S-2399	334.2	238.2, (258.1)	200	111	10	37, (27)	18, (20)
S-2399-d_3	337.1	260.9	200	111	10	274	18

( ) = confirmatory ion

APPENDIX 3 CALCULATIONS

The following example uses data generated from batch "VP-39058- Trial 1_24Nov2015" to calculate the curve data and 3′-OH-S-2840 percent recovery for Sample #4 (untreated control + LOQ).

Peak integration was performed by Analyst® software version 1.6.2. The concentrations of the analytes (S-2399, 3′-0H-S-2840, 1′-COOH-S-2840A, and 1′- COOH-S-2840B) in each sample extract were calculated using a second-order polynomial equation on the basis of the MS detector peak area ratio (analyte peak area response/internal standard peak area response). The data were presented graphically as concentration of the calibration standards verses their peak area ratios using an Excel® spreadsheet to determine the curve parameters and calculate the sample residues.

The data were weighted relative to the concentration of the highest standard (the largest calibration standard concentration was divided by a calibration standard concentration to get the number of entries in a data set). The calibration standard set, with the number of data entries is shown below.

Standard Concentration	Number of Entries in Data Set	3′-OH-S-2840 Peak Area Ratio
10.0 µg/L	1	9.53
5.00 µg/L	2	4.78
2.50 µg/L	4	2.38
1.00 µg/L	10	0.911
0.500 µg/L	20	0.469
0.250 µg/L	40	0.243

The peak area ratios were entered as the *x* values and concentrations as the *y* values to calculate a curve expressed as the following equation:

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

A = -0.00110
B = 1.06
C = 0.00100

The peak area ratios of the calibration standards were entered into this equation and the standard concentrations calculated. For example (from the above data), the 2.50 µg/L standard has a peak area ratio of 2.38 has a calculated concentration using the equation as follows:

$$Y = (-0.00110 \times 2.38 \times 2.38) + (1.06 \times 2.38) + 0.00100 = 2.52 \text{ µg/L}$$

Sample extract concentrations of each analyte were also calculated as above except the average peak area ratio of the control samples was subtracted from the peak area ratio of the fortified sample. For example, Sample #4 extract was fortified with 0.0100 ppm 3′-OH-S-2840 with a peak area ratio of 0.439 and an average control area ratio of 0.0239 has a concentration as follows:

$$[-0.00110 \times (0.439-0.0239) \times (0.439-0.0239)] + [1.06 \times (0.439-0239)] + 0.00100 = 0.441\ \mu g/L$$

The concentration of each analyte (S-2399, 3′-OH-S-2840, 1′-COOH-S-2840-A, and 1′-COOH- S-2840-B) in the fortified sample was calculated using the following formula:

$$ppm = \frac{C \times EV \times FV \times DF \times (1/1000)}{W \times AV}$$

where

C = concentration of extract (in μg/L, from equation) EV = total extraction volume (100 mL)
FV = final volume of extract (2.00 mL)
DF = dilution factor (if any)
W =sample weight analyzed (10.00 g)
AV =aliquot volume (1.00 mL)

For example, the concentration of 3′-OH-S-2840 in Sample #4 (with a calculated extract concentration of 0.441 μg/L) was calculated as follows:

$$ppm = \frac{0.441\ \mu g/L \times 100\ mL \times 2.00\ mL \times (1\ L/1000\ mL)}{10.00\ g \times 1.00\ mL} = 0.00882\ \mu g/g$$

Recoveries were calculated using the following equation:

$$\text{Recovery (\%)} = \frac{\text{ppm in fortified sample}}{\text{Fortification level}} \times 100$$

For example, the percent recovery of 3′-OH-S-2840 in Sample #4 (with a calculated sample concentration of 0.00882 μg/g) was calculated as follows:

$$\text{Recovery (\%)} = \frac{0.00882 \text{ ppm}}{0.0100 \text{ ppm}} \times 100 = 88.2\%$$

Recovery results of each analyte were calculated by multi-point calibration for each sample. A statistical treatment of the data includes the calculation of means, standard deviations (SD), RSDs as percentages (%), and the 95% confidence intervals. All statistics were calculated using Microsoft® Office Excel 2010.