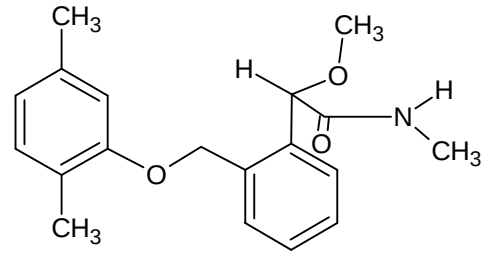
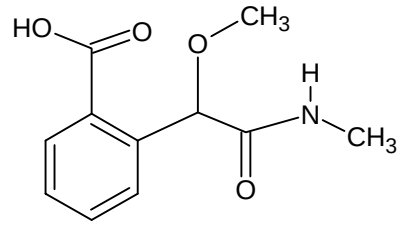
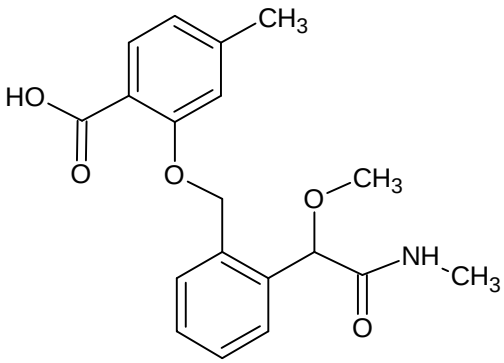
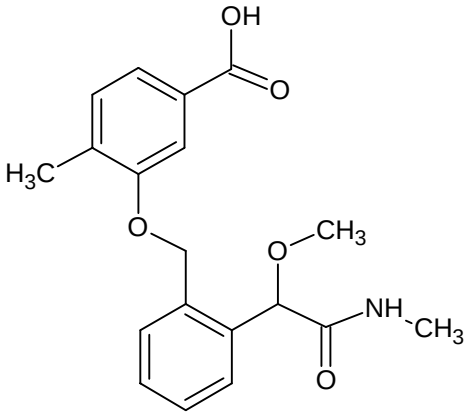
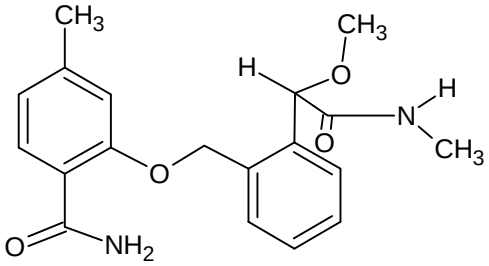
Analytical Reference Standard

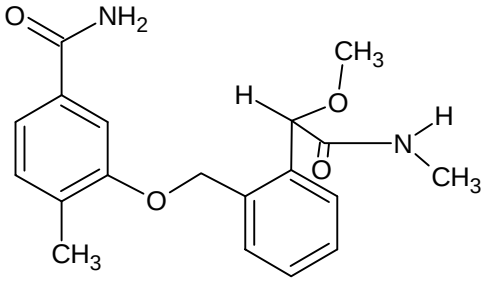
Standard Name:	S-2200 (V-10190)
Lot Number:	AS 2261c
IUPAC Name:	(*RS*)-2-[(2,5-dimethylphenoxy)methyl]-α-methoxy-*N*-methyl-benzeneacetamide
Sample Archive No.:	V-Arc-2158
Manufacturer's ID:	Valent Reference VTC-1268-29
Purity:	99.6%
Molecular Formula:	$C_{19}H_{23}NO_3$
Average Mass:	313.4
Molecular Structure:	

Standard Name:	DX-CA-S-2200
Lot Number:	AS 2269c
IUPAC Name:	(*RS*)-2-(*N*-methylcarbamoyl-methoxymethyl)benzoic acid
Sample Archive No.:	V-Arc-2307
Manufacturer's ID:	Sumitomo Lot 12SC8283515
Purity:	99.7%
Molecular Formula:	$C_{11}H_{13}NO_4$
Average Mass:	223.2
Molecular Structure:	

Standard Name:	2-COOH-S-2200
Lot Number:	AS 2268b
IUPAC Name:	(*RS*)-2-{2-[1-methoxy-1-(*N*-ethylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzoic acid
Sample Archive No.:	V-Arc-2255
Manufacturer's ID:	Sumitomo Lot 317-001-47-1
Purity:	98.9%
Molecular Formula:	$C_{19}H_{21}NO_5$
Average Mass:	343.4
Molecular Structure:	

Standard Name:	5-COOH-S-2200
Lot Number:	AS 2267b
IUPAC Name:	(*RS*)-3-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzoic acid
Sample Archive No.:	V-Arc-2254
Manufacturer's ID:	Sumitomo Lot 262-005-10-1
Purity:	96.6%
Molecular Formula:	$C_{19}H_{21}NO_5$
Average Mass:	343.4
Molecular Structure:	

Standard Name:	2-$CONH_2$-S-2200
Lot Number:	AS 2318b
IUPAC Name:	(*RS*)-2-{2-{1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzamide
Sample Archive No.:	V-Arc-2261
Manufacturer's ID:	Sumitomo Lot CTS11005
Purity:	95.2%
Molecular Formula:	$C_{19}H_{22}N_2O_4$
Average Mass:	342.4
Molecular Strucutre:	

Standard Name:	5-$CONH_2$-S-2200
Lot Number:	AS 2319a
IUPAC Name:	(*RS*)-3-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzamide
Sample Archive No.:	V-Arc-2213
Manufacturer's ID:	Sumitomo Lot CTS10012
Purity:	95.3%
Molecular Formula:	$C_{19}H_{22}N_2O_4$
Average Mass:	342.4
Molecular Structure:	

Other

Upon completion of the study, a copy of the protocol and the final report will be archived at CPS. The original protocol, final report, raw data, correspondence, and other documentation will be transferred to the Valent Archives, Valent U.S.A. Corporation, 6560 Trinity Court, Dublin, California, 94568.

1.0 EXECUTIVE SUMMARY

Valent Method RM-48S-3, entitled "Determination of S-2200 and Metabolites in Soil" [1], was validated successfully in Trial 2. 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].

The method was successfully validated on the second attempt for S-2200 and its five metabolites (DX-CA-S-2200, 2-COOH-S-2200, 5-COOH-S-2200, 2-$CONH_2$-S-2200, and 5-$CONH_2$-S-2200) in soil samples at the limit of quantitation (LOQ) and at 10×LOQ concentration levels (0.020 ppm and 0.200 ppm, respectively). The first attempt was rejected because the recovery of S-2200 in one of the samples (fortified at the LOQ level) fell outside the acceptable range of 70 to 120%. The five metabolites had acceptable results for Trial 1.

The method was performed as written with no major modifications. It took one person approximately 8.0 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 12.5 hours. To complete one set, including extraction and analysis, took approximately 2.6 days.

This method passed the independent laboratory validation (ILV) on the second attempt with no major modifications.

2.0 INTRODUCTION

The objective of this study was to validate Valent Method RM-48S-3, entitled "Determination of S-2200 and Metabolites in Soil" [1]. This method was successful.

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 Substance

Standard name: S-2200 (V-10190)
Lot Number: AS 2261c
IUPAC name: (*RS*)-2-[(2,5-dimethylphenoxy)methyl]-α-methoxy-*N*-methyl-benzeneacetamide
Sample archive no.: V-Arc-2158
Manufacturer's ID: Valent Reference VTC-1268-29
Purity: 99.6%
Date of analysis: 07 May 2012
Expiration date: 07 May 2013
Storage conditions: Frozen

Standard name: DX-CA-S-2200
Lot Number: AS 2269c
IUPAC name: (*RS*)-2-(*N*-methylcarbamoyl-methoxymethyl)benzoic acid
Sample archive no.: V-Arc-2307
Manufacturer's ID: Sumitomo Lot 12SC8283515
Purity: 99.7%
Date of analysis: 29 May 2012
Expiration date: 29 May 2013
Storage conditions: Frozen

Standard name: 2-COOH-S-2200
Lot Number: AS 2268b
IUPAC name: (*RS*)-2-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzoic acid
Sample archive no.: V-Arc-2255
Manufacturer's ID: Sumitomo Lot 317-001-47-1
Purity: 98.9%
Date of analysis: 30 July 2012
Expiration date: 30 July 2013
Storage conditions: Frozen

Standard name: 5-COOH-S-2200
Lot Number: AS 2267b
IUPAC name: (*RS*)-3-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzoic acid
Sample archive no.: V-Arc-2254
Manufacturer's ID: Sumitomo Lot 262-005-10-1
Purity: 96.6%
Date of analysis: 30 July 2012
Expiration date: 30 July 2013
Storage conditions: Frozen

Standard name:	2-CONH2-S-2200
Lot Number:	AS 2318b
IUPAC name:	(*RS*)-2-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzamide
Sample archive no.:	V-Arc-2261
Manufacturer's ID:	Sumitomo Lot CTS11005
Purity:	95.2%
Date of analysis:	17 July 2012
Expiration date:	17 July 2013
Storage conditions:	Frozen

Standard name:	5-CONH2-S-2200
Lot Number:	AS 2319a
IUPAC name:	(*RS*)-3-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]-benzyloxy}-4-methyl-benzamide
Sample archive no.:	V-Arc-2213
Manufacturer's ID:	Sumitomo Lot CTS10012
Purity:	95.3%
Date of analysis:	07 February 2012
Expiration date:	07 February 2013
Storage conditions:	Frozen

3.2 Test System

The test system used for the validation was an untreated soil sample obtained from Georgia by Valent U.S.A. Corporation. The sample was stored at room temperature until needed for analysis.

3.3 Equipment and Reagents

The equipment and reagents used for the method validation were as outlined in Valent Method RM-48S-3 for Trial 1 and Trial 2 (in Appendix 5, on page 1: Reagents and on page 4: Equipment). Identical or equivalent apparatus and materials were used.

3.3.1 Equipment and Apparatus

Allegra™ X-22R Centrifuge (Beckman Coulter®)
Analytical Balance (Mettler Toledo)
Manual Micro Pipettor 200 µL (VWR International)
Manual Micro Pipettor 1000 µL (VWR International)
Manual Micro Pipettor 5000 µL (VWR International)
Recirculating Chiller (Polystat K6-S3)
Refrigerator/Freezer (Nor-lake® Scientific)
Rotavapor® R-124 (Büchi)
Top-loading Balance (Mettler Toledo)

Ultrasonic Cleaner 5210 (Branson)
Ultrasonic Cleaner 5510 (Branson)
Vacuum Controller V-850 (Büchi)
Vacuum Pump V-700 (Büchi)
Wrist Action® Shaker Model 75 (Burrell)

3.3.2 Reagents

Acetone (Pharmco-AAPER)
Dichloromethane (EMD)
Formic Acid (Sigma-Aldrich®)
HPLC-grade Water (EMD)
Hydrochloric Acid (EMD)
Methanol (EMD)
Sodium Chloride (J.T. Baker®)

3.4 Experimental Design

3.4.1 Establishment of the Method

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

3.4.2 Sample Validation Sets, Fortification, and Extraction Procedure

Sample Validation Sets
Each analytical set consisted of 13 samples: one reagent blank, two untreated controls, five untreated controls fortified with S-2200 and its five metabolites at the LOQ (0.0200 ppm), and five untreated controls fortified with S-2200 and its five metabolites at 10×LOQ (0.200 ppm).

Data are summarized in Table 1 to Table 6 for Trial 1, and in Table 7 to Table 12 for Trial 2. Residue data sheets are included in Appendix 1.

Calibration standard solutions (1.25 to 50.0 ng/mL) and blanks were also included in each sample set.

The estimated limit of detection (LOD) for each analyte is 0.01 ppm (see Appendix 5, page 9: Limits of Detection and Quantitation).

Fortification

The LOQ and 10×LOQ samples were fortified with 50.0 µL of the appropriate fortification standard solutions of S-2200 and its five metabolites. The fortification standard solutions had a concentration of 1.00 µg/mL for the LOQ and a concentration of 10.0 µg/mL for the 10×LOQ.

Extraction and Workup

The following extraction steps were followed for each sample.

1. Using a top-loading balance, weighed 2.5 g of control sample into a tared 50-mL polypropylene tube.
2. Added the appropriate amount of fortification solution to the sample.
    a. For the reagent blank and the untreated controls, added 0 µL.
    b. For the LOQ samples, added 50.0 µL of 1.00 µg/mL S-2200 and its five metabolites fortification solution.
    c. For the 10×LOQ samples, added 50.0 µL of 10.0 µg/mL S-2200 and its five metabolites fortification solution.
3. Added 25 mL of (80:20, v/v) acetone/0.05 M hydrochloric acid in HPLC-grade water solution to each sample, and vortexed.
4. Placed tubes horizontally on a wrist-action shaker, and shook for 60 minutes.
5. Centrifuged samples at 2500 rpm for 5 minutes.
6. Decanted entire extract into a 250-mL glass separatory funnel.
7. Added 25 mL of (80:20, v/v) acetone/0.05 M hydrochloric acid in HPLC-grade water solution to each sample, and vortexed.
8. Placed tubes horizontally on a wrist-action shaker, and shook for 60 minutes.
9. Centrifuged samples at 2500 rpm for 5 minutes.
10. Decanted entire extract into the appropriate separatory funnel, combining extracts 1 and 2. For the reagent blank, added 100 mL of (80:20, v/v) acetone/0.05 M hydrochloric acid in HPLC-grade water solution into a 250-mL glass separatory funnel.
11. Added 50 mL of 5% (w/v) sodium chloride in HPLC-grade water solution and 50 mL of dichloromethane to each separatory funnel.
12. Hand shook each separatory funnel for 1 minute, and allowed the layers to separate.
13. Drained the organic layer (the lower layer) into a 500-mL round-bottom flask.
14. Added another 50 mL of dichloromethane to each separatory funnel.
15. Hand shook each separatory funnel for 1 minute, and allowed the layers to separate.
16. Drained the organic layer (the lower layer) into the appropriate 500-mL round-bottom flask, combining partitions 1 and 2. Discarded the aqueous layer.
17. Evaporated the partitions to dryness using a rotary evaporator with a water bath set at ≤40°C.
18. Added 20 mL of (50:50, v/v) 0.05% formic acid in HPLC-grade water/0.05% formic acid in methanol solution to each of the round-bottom flasks, and sonicated briefly to dissolve the residues.
19. Transferred a 1-mL aliquot of sample to an HPLC vial to be analyzed by

LC-MS/MS.

3.4.3 Sample Processing and Analysis

The samples were processed and analyzed as described by Valent Method RM-48S-3 [1] for Trial 1 and Trial 2.

3.4.4 Fortification and Calibration Standard Solutions Preparation

Trial 1 and Trial 2
The primary stock solution for each reference standard was prepared by weighing approximately 20.0 mg of compound onto a tared piece of weigh paper and transferring to a 100-mL glass volumetric. Acetone was added up to volume and sonicated appropriately.

A fortification solution, containing S-2200 and its five metabolites, was prepared at a concentration of 10.0 µg/mL (each analyte) by adding an appropriate amount of each primary stock into a 10.0-mL volumetric flask and diluting up to volume with acetone. A second fortification solution was prepared at a concentration of 1.00 µg/mL (each analyte) by measuring 1.00 mL of the initial fortification solution into a 10-mL volumetric flask and diluting to volume with acetone.

A secondary stock solution, containing S-2200 and its five metabolites, was prepared at a concentration of 100 ng/mL (each analyte) by adding an appropriate amount of each primary stock solution to a 100-mL volumetric flask and diluting to volume with (50:50, v/v) 0.05% formic acid in HPLC-grade water/0.05% formic acid in methanol solution.

The calibration standard solutions were prepared at concentrations ranging from 1.25 to 50.0 ng/mL by adding an appropriate amount of secondary stock solution to an HPLC vial and diluting to a total volume of 1 mL (50:50, v/v) 0.05% formic acid in HPLC-grade water/0.05% formic acid in methanol solution. Calibration standard solutions were prepared fresh daily.

All storable solutions were refrigerated (4°C) when not in use.

3.5 LC-MS/MS Instrumentation

Instrumentation
Agilent 1200® HPLC System (Agilent Technologies)
API 4000™ Tandem Mass Spectrometer, MS/MS (Applied Biosystems®)
HPLC Column: Agilent Eclipse XDB-C18 150 × 4.6 mm, 5 µm
(Agilent Technologies)
Software: Applied Biosystems®, Analyst® 1.5.1

3.6 Data Acquisition and Reporting

Peak integration was performed by Analyst® software version 1.5.1. The MS detector responses (peak area) for various injected standard concentrations were used to generate an external calibration curve for the analytes of interest. The overall purpose for the external calibration curve was to display acceptable linearity ($r^2 \geq 0.99$) of the assigned calibration range. The recoveries of the analytes from the fortified samples were calculated by multi-point calibration.

Recovery results of each analyte were computed for each sample. The equations used for quantification are presented in Appendix 2. 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 2003.

4.1 Method Establishment

The S-2200, DX-CA-S-2200, 2-COOH-S-2200, 5-COOH-S-2200, 2-$CONH_2$-S-2200, and 5-$CONH_2$-S-2200 primary transitions (from m/z 314.20 to 192.20, m/z 224.10 to 146.10, m/z 344.20 to 192.20, m/z 344.20 to 192.20, m/z 343.20 to 192.20, and m/z 343.20 to 192.20, respectively) were used for quantitation.

Prior to performing the ILV, the analyte retention times, instrument detection limits, and linearity of instrument responses to a range of analyte concentrations were determined, and the test system was verified as free of interferences at appropriate retention times.

TABLE 13 HPLC SYSTEM OPERATING PARAMETERS

HPLC System: Agilent Model 1200®
Software: Applied Biosystems®, Analyst® 1.5.1
HPLC Column: Agilent Eclipse XDB-C18 150 × 4.6 mm, 5 µm
Column Temperature: 20°C
Injection Volume: 10.0 µL
Run Time: 30.0 minutes

Mobile Phase: (A—Aqueous): 0.05% formic acid in HPLC-grade water
(B—Organic): 0.05% formic acid in methanol
Needle Wash: (50:50, v/v) HPLC-grade water/methanol
Wash Time: Flush Port, 0.25 minutes

Gradient:

Time (min)	A (%)	B (%)	Flow (µL/min)
0.00	50.0	50.0	500
2.00	50.0	50.0	500
8.00	10.0	90.0	500
9.00	10.0	90.0	500
9.50	10.0	90.0	250
13.00	10.0	90.0	250
13.50	10.0	90.0	500
20.00	10.0	90.0	500
22.50	50.0	50.0	500
30.00	50.0	50.0	500

TABLE 14 MS/MS OPERATING PARAMETERS

Tandem Mass Spectrometry System, Applied Biosystems®, API 4000™
Software: Applied Biosystems®, Analyst® 1.5.1

The following parameters were used for operation of the mass spectrometer:

Parameter	Setting
Ion Source:	TurboSpray
Scan Type:	MRM
Polarity:	Positive
Curtain Gas (CUR):	40.00
Temperature (TEM):	500.00
Ion Spray Voltage (IS):	5500
Collision Gas (CAD):	7.00
Ion Source Gas 1 (GS1):	50.00
Ion Source Gas 2 (GS2):	50.00
Interface Heater (ihe):	ON
Declustering Potential (DP):	40.00
Entrance Potential (EP):	10.00
Analyte	S-2200
Transitions Monitored:	(Q1) 314.20→(Q3) 192.20 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	16.00
Collision Cell Exit Potential(CXP):	12.00
Analyte	DX-CA-S-2200
Transitions Monitored:	(Q1) 224.10→(Q3) 146.10 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	29.00
Collision Cell Exit Potential(CXP):	9.00
Analyte	2-COOH-S-2200
Transitions Monitored:	(Q1) 344.20→(Q3) 192.20 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	16.00
Collision Cell Exit Potential(CXP):	12.00
Analyte	5-COOH-S-2200
Transitions Monitored:	(Q1) 344.20→(Q3) 192.20 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	16.00
Collision Cell Exit Potential(CXP):	12.00
Analyte	2-CONH2-S-2200
Transitions Monitored:	(Q1) 343.20→(Q3) 192.20 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	16.00
Collision Cell Exit Potential(CXP):	12.00
Analyte	5-CONH2-S-2200
Transitions Monitored:	(Q1) 343.20→(Q3) 192.20 m/z
Dwell Time (msec):	150.00
Collision Energy (CE):	16.00
Collision Cell Exit Potential(CXP):	12.00

APPENDIX 2 CALCULATIONS

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystems®, Analyst® software version 1.5.1 using a second order polynomial regression with 1/concentration weighting. Further calculations were performed using the software Microsoft® Office Excel 2003.

The quadratic equation is expressed as:

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

where

y = Concentration (ng/mL)
x = Native peak area

By means of the quadratic equation, the content of S-2200 and metabolites in soil or recoveries can be calculated as follows:

$$\text{Extraction Concentration (ng/mL)} = Ax^2 + Bx + C$$

The residue S-2200 and metabolites in test samples is calculated as follows:

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

where

C = Concentration of extract (in ng/mL)
FV = Final volume of extract = (20 mL)
DF = Dilution factor (if diluted after final volume) = (1)
W = Sample weight analyzed = (2.5 g)

Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R)}{T} \times 100$$

where

R = ppm of target analyte found in fortified sample
T = Theoretical ppm in fortified sample