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TITLE

V-10456, S-3100-CA, and S-3100-CA-UR - Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions

TEST GUIDELINE

860.1340

AUTHOR(S)

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STUDY COMPLETION DATE

2021-02-16

PERFORMING LABORATORY

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LABORATORY PROJECT ID

12709.6509

TOTAL PAGES

83

1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of V-10456 and its metabolites, S-3100-CA and S-3100-CA-UR, in aqueous solution. The method was validated (27 April to 1 May 2020) to quantify the concentrations of V-10456, S-3100-CA, and S-3100-CA-UR present in recovery samples prepared in dilute, natural, filtered seawater. The analytical method was validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of analyte identification.

The method was validated by fortification of dilute, natural, filtered seawater with V-10456 and its metabolites, S-3100-CA and S-3100-CA-UR, at concentrations summarized in the table below.

Test Substance	Test Concentrations (µg/L)	
	LOQ	High
V-10456	0.100	1000
S-3100-CA	1.00	1000
S-3100-CA-UR	1.00	1000

Recovery samples were diluted with 56/44/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) to a final composition of 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v). The High-level recovery samples were further diluted into the calibration range with 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 22 April 2020, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the validation was conducted on 27 April to 1 May 2020 at Smithers, located in Wareham, Massachusetts. All original raw data, the protocol, and a copy of the final report produced during this study are stored in Smithers' at the above location.

2.0 MATERIALS AND METHODS

2.1 Protocol

Procedures used in this study followed those described in the Smithers Viscient protocol entitled "V-10456, S-3100-CA, and S-3100-CA-UR – Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions" (Appendix 1). The study was conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160 (U.S. EPA, 1989) and the OECD principles on GLP (OECD, 1998), and followed the guidance documents SANCO/3029/99 rev. 4 (EC, 2000) and OCSPP 860.1340 (U.S. EPA, 1996).

2.2 Test and Reference Substances

2.2.1 Test Substances

The test substance, S-3100 TG, was received on 26 January 2018 from Valent Technical Center, Dublin, California. The following information was provided (Certificate of Analysis, Appendix 2):

Name:	S-3100 TG
Synonym:	V-10456 technical
Lot No.:	17TK11S3G
CAS No.:	Not Listed
Purity:	96.8%
Expiration Date:	23 November 2020

Upon receipt at Smithers Viscient, the test substance (SMV No. 9268) was stored at room temperature in a dark, ventilated cabinet in a 1-L Nalgene bottle. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples during testing.

The test substance, S-3100-CA, was received on 20 February 2020 from Sumitomo Chemical Co., Ltd., Hyogo, Japan. The following information was provided (Certificate of Analysis, Appendix 2):

Name:	S-3100-CA
Lot No.:	19SC8673272
CAS No.:	Not Listed
Purity:	98.6%
Expiration Date:	30 November 2022

Upon receipt at Smithers, the test substance (TMC No. 10518) was stored refrigerated in the original container. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples and calibration standards during testing.

The test substance, S-3100-CA-UR, was received on 24 April 2019 from Valent USA, LLC, Dublin, California. The following information was provided (Certificate of Analysis, Appendix 2):

Name:	S-3100-CA-UR
Lot No.:	AS 2434a
CAS No.:	Not Listed
Purity:	97.1%
Expiration Date:	5 November 2021

Upon receipt at Smithers Viscient, the test substance (SMV No. 10007) was stored at in a freezer in the original container. Concentrations were adjusted for the purity of the test substance. This sample of test substance was used to prepare recovery samples and calibration standards during testing.

2.2.2 Reference Substance

The reference substance, V-10456, was received on 21 April 2020 from Valent USA LLC, Dublin, California. The following information was provided (Certificate of Analysis, Appendix 2):

Name:	V-10456
Synonym(s):	V-10456 analytical standard
Lot No.:	AS 2429b
CAS No.:	Not Listed
Purity:	99.4%
Expiration Date:	3 April 2021

Upon receipt at Smithers, the reference substance (TMC No. 10624) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the reference substance. This sample of reference substance was used to prepare calibration standards during testing.

Determination of stability and characterization, verification of the test and reference substance identities, maintenance of records on the test and reference substances, and archival of a sample of the test and reference substances are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	EMD, Honeywell, reagent grade
2.	Methanol:	EMD, reagent grade
3.	Formic acid:	BDH, Honeywell, reagent grade
4.	0.1% formic acid in acetonitrile:	Fisher, reagent grade
5.	Purified reagent water:	Fisher, Prepared from a Millipore MilliQ Direct 8 water purification system (meets ASTM Type II requirements)

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu SIL-20ACXR autoinjector Shimadzu DGU-20A5R vacuum degassers Shimadzu LC-20ADXR solvent delivery pumps Shimadzu CTO-20AC column compartment Shimadzu CBM-20A communications bus Analyst 1.6.3 software for data acquisition
2.	Balance:	Mettler Toledo XSE205DU
3.	Laboratory Equipment:	Positive displacement pipets, Erlenmeyer flasks, stir bars, stir plates, vortexer, sonicator, beakers, volumetric flasks, disposable glass vials, disposable glass pipets, graduated cylinders, Pasteur pipets, autosampler vials, and amber glass bottles with Teflon-lined caps

Other equipment or instrumentation may be used in future testing but may require optimization to achieve the desired separation and sensitivity.

2.5 Test Matrix

Dilute, natural, filtered seawater was used as dilution and control water during this study. Natural seawater was pumped from the Cape Cod Canal, Bourne, Massachusetts. The water was collected at about 1 to 4 meters offshore and a depth of approximately 0.5 meters. The seawater was then transferred by a pump (fiberglass reinforced thermoplastic housing) through polyvinyl chloride (PVC) pipes and transported to the laboratory in a 6080-L polyethylene holding tank. The water was diluted to a salinity of 20 ± 3% with on-site laboratory well water, which was a mixture of unadulterated water from a 100-meter bedrock well and de-chlorinated Town of Wareham well water. The water was then filtered through 50-µm and 1-µm polypropylene bag filters, and heated to the required test temperature.

2.6 Preparation of Liquid Reagents

The volumes listed in this section were those used during the validation. For future testing, the actual volumes used may be scaled up or down as necessary.

A 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) liquid reagent solution was typically prepared by combining 120 mL of acetonitrile, 120 mL purified reagent water, and 0.240 mL of formic acid. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 56/44/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) liquid reagent solution was typically prepared by combining 560 mL of acetonitrile, 440 mL of purified reagent water, and 1.00 mL of formic acid. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v) liquid reagent solution was typically prepared by combining 500 mL of acetonitrile, 100 mL of dilute, natural, filtered seawater, 400 mL of purified reagent water, and 1.00 mL of formic acid. The solution was mixed well using a stir bar and stir plate for 5 minutes.

A 50/50 acetonitrile/purified reagent water (v/v) liquid reagent solution was typically prepared by combining 150 mL of acetonitrile and 150 mL of purified reagent water. The solution was mixed well prior to use.

A 0.1% formic acid in ultra-purified reagent water mobile phase solution was typically prepared by adding 4.00 mL of formic acid to 4000 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for 5 minutes, then degassed under vacuum with sonication for 10 minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was typically prepared by combining 1500 mL of acetonitrile, 1500 mL of methanol, and 2000 mL of purified reagent water. The solution was mixed well before use.

2.7 Preparation of Stock Solutions

The volumes and masses listed in this section were those used during the validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

Primary stock solutions were typically prepared as described in the table below:

Primary Stock ID	Amount Weighed (g), Net Weight	Amount Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
Test substance						
9268-1BC	0.0517	0.0500	Acetonitrile	50.0	1000	Secondary stock and sub-stock solutions
10518H	0.0508	0.0501	50/50 acetonitrile/purified reagent water (v/v)	50.0	1000	Secondary stock and sub-stock solutions
10007D	0.01034	0.0100	50/50 acetonitrile/purified reagent water (v/v)	10.0	1000	Secondary stock and sub-stock solutions
Reference substance						
10624A	0.01009	0.01003	Acetonitrile	10.0	1000	Secondary stock solutions
10518G	0.0509	0.0502	50/50 acetonitrile/purified reagent water (v/v)	50.0	1000	Secondary stock solutions

Secondary stock solutions were typically prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
Test substance							
9268-1BC	1000	0.500	50.0	Acetonitrile	9268-1BC-1	10.0	Sub-stock solutions
10518H	1000	0.500	50.0	50/50 acetonitrile/purified reagent water (v/v)	10518H-1	10.0	Sub-stock solutions
10007D	1000	0.500	10.0	50/50 acetonitrile/purified reagent water (v/v)	10007D-1	10.0	Sub-stock solutions
Reference substance							
10624A	1000	0.500	50.0	Acetonitrile	10624A-1	10.0	Sub-stock solutions
10518G	1000	0.500	50.0	50/50 acetonitrile/purified reagent water (v/v)	10518G-1	10.0	Sub-stock solutions

Sub-stock solutions were typically prepared as per the table below:

Fortifying Stock ID	Fortifying Stock Concentration (mg/L)	Volume of Fortification (mL)	Final Volume (mL)	Stock Solvent	Stock ID	Stock Concentration (mg/L)	Stock Use
Test substance							
9268-1BC-1	10.0	0.0200	20.0	50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v)	Mixed Tech Stk 1	0.0100	LOQ-recovery samples
10007D-1	10.0	0.200				0.100	
10518H-1	10.0	0.200				0.100	
9268-1BC	1000	0.200	20.0		Mixed Tech Stk 2	10.0	High-level recovery samples
10007D	1000	0.200				10.0	
10518H	1000	0.200				10.0	
Reference substance							
10624A-1	10.0	0.0200	20.0	50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v)	Mixed Ana 1	0.0100	Calibration standards
10007D-1	10.0	0.100				0.0500	
10518G-1	10.0	0.100				0.0500	

All primary and secondary stock solutions were stored refrigerated (2 to 8 °C) in amber glass bottles fitted with Teflon-lined caps. Sub-stock solutions were prepared fresh on the day of use and discarded after use.

2.8 Preparation of Calibration Standards

Calibration standards were prepared in 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v) by fortifying with the Mixed Ana 1 sub-stock solution (0.0100/0.0500/0.0500 mg/L as V-10456/S-3100-CA/S-3100-CA-UR) to yield concentrations of 0.00500, 0.0100, 0.0200, 0.0300, 0.0400, and 0.0500 µg/L for V-10456 and 0.0250, 0.0500, 0.100, 0.150, 0.200, and 0.250 µg/L for S-3100-CA and S-3100-CA-UR.

2.9 Matrix Effects Investigation

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based standards in the following manner. Calibration standards used to assess possible matrix effects were prepared in triplicate in 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v) and 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) by fortifying with the appropriate sub-stock solution, as outlined in the tables below. Resulting standards had concentrations of 0.0100 µg/L as V-10456, and 0.0500 µg/L as S-3100-CA and S-3100-CA-UR.

Stock ID	Stock Concentration[a] (mg/L)	Sample ID	Sample Type	Volume of Fortification (mL)	Final Volume (mL)	Fortified Concentration[a] (µg/L)
Mixed Ana Stk 1	0.0100/ 0.0500/ 0.0500	MM-Std A, B, & C	Matrix-matched standards	0.0500	50.0[b]	0.0100/0.0500/0.0500
Mixed Ana Stk 1	0.0100/ 0.0500/ 0.0500	Sol-Std A, B, & C	Solvent standards	0.0500	50.0[c]	0.0100/0.0500/0.0500

[a] Concentrations expressed as concentration V-10456/S-3100-CA/S-3100-CA-UR.
[b] Diluent: 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v)
[c] Diluent: 50/50/0.1 acetonitrile/purified reagent water/formic acid (v/v/v)

2.10 Sample Fortification and Preparation

The recovery samples were prepared in dilute, natural, filtered seawater by fortification with the appropriate sub-stock solution to yield samples with concentrations of 0.100, 1.00, 1.00 µg/L

(LOQ) and 1000, 1000, and 1000 µg/L (High) as V-10456, S-3100-CA and S-3100-CA-UR, respectively. The original S-3100-CA-UR High-level results failed to meet acceptance criteria for the original analysis. The High-level samples for S-3100-CA-UR were prepared on a separate occasion. It is these results that are presented here. Recovery samples were prepared separately ("de novo") at these concentrations. Five replicates were prepared for each concentration level. Additionally, five samples were left unfortified to serve as controls and were diluted in the same fashion as the LOQ concentration recovery samples. In addition, two reagent blank samples were prepared and processed in the same manner as the control samples. The preparation procedure is outlined in the tables below.

Sample ID 12709-6509-	Sample Type	Stock Concentration[a] (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration[a] (µg/L)
01 & 02	Reagent Blank	NA[b]	NA	5.00	0.00
03, 04, 05, 06, & 07	Control	NA	NA	5.00	0.00
08, 09, 10, 11, & 12	LOQ	0.0100/ 0.100/ 0.100	0.0500	5.00	0.100/ 1.00/ 1.00
13, 14, 15, 16, & 17	High[c]	10.0/ 10.0/ 10.0	0.500	5.00	1000/ 1000/ 1000

[a] Concentrations expressed as concentration V-10456/S-3100-CA/S-3100-CA-UR.
[b] NA = Not Applicable.
[c] Sample ID's for S-3100-CA-UR samples were 12709-6509-18 to 22. Please see discussion above.

2.11 Dilution of Samples

To minimize the potential for losses of the test substance during processing, the aqueous recovery samples were not sub-sampled prior to dilution. The first dilution with 56/44/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) was performed by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v). The High-level recovery samples were subsequently diluted into the calibration standard range with of 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v). Due to the different concentrations of the calibration standards, two separate dilutions of the High-level samples were analyzed. The two different dilutions are presented in the table below as -2 and -3 replicates. The dilution procedure is outlined in the table below.

Sample ID 12709-6509-	Sample Type	Nominal Concentration[a] (µg/L)	Sample Volume (mL)	Final Volume[b] (mL)	Sample Volume (mL)	Final Volume[c] (mL) -2	Sample Volume (mL)	Final Volume[b] (mL) -3	Dilution Factor[d] -2/-3
01 & 02	Reagent Blank	0.00	5.00	50.0	NA[e]	NA	NA	NA	10.0
03, 04, 05, 06, & 07	Control	0.00	5.00	50.0	NA	NA	NA	NA	10.0
08, 09, 10, 11, & 12	LOQ	0.100/ 1.00/ 1.00	5.00	50.0	NA	NA	NA	NA	10.0
13, 14, 15, 16, & 17	High[f]	1000/ 1000/ 1000	5.00	50.0	0.0900	50.0	1.00	5.00	5560/ 27,800

[a] Concentrations expressed as concentration V-10456/S-3100-CA/S-3100-CA-UR
[b] Dilution solvent: 56/44/0.1 acetonitrile/purified reagent water/formic acid (v/v/v)
[c] Dilution solvent: 50/10/40/0.1 acetonitrile/dilute, natural, filtered seawater/purified reagent water/formic acid (v/v/v/v)
[d] The-2 samples were analyzed for S-3100-CA and S-3100-CA-UR and the -3 samples were analyzed for V-10456.
[e] NA = Not Applicable
[f] Sample ID's for S-3100-CA-UR samples were 12709-6509-18 to 22. Please see discussion above.

2.12 Analysis

2.12.1 Instrumental Conditions

The LC-MS/MS analysis was conducted utilizing the following instrumental conditions:

LC parameters:

Column: Phenomenex Kinetex Phenyl-hexyl 2.6 µm, 3 × 50 mm
Mobile Phase A: 0.1% formic acid in ultra-purified reagent water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.01	0.500	90.0	10.0
0.50	0.500	90.0	10.0
4.00	0.500	0.00	100
4.50	0.500	0.00	100
4.60	0.500	90.0	10.0
6.00	0.500	90.0	10.0

Run Time: 6.0 minutes
Injector Rinse Solvent: 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature: 40 °C
Sample Temperature: 10 °C
Injection Volume: 35.0 µL
Retention Time: approximately 4.0 minutes (V-10456)
approximately 3.6 minutes (S-3100-CA)
approximately 3.0 minutes (S-3100-CA-UR)

MS parameters:

Instrument:	MDS Sciex API 5000 mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Source Temperature:	650 °C
Curtain Gas:	20.0
Ion Source – Gas 1 / Gas 2:	50.0 / 50.0
Collision Gas:	8.00
Resolution (Q1/Q3):	Unit/Low

Test Substance	V-10456		S-3100-CA		S-3100-CA-UR	
Instrument Parameter	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	518.1/472.1	518.1/444.1	490.2/249.9	490.2/292.9	370.2/312.9	370.2/351.7
Dwell Time (milliseconds):	100	100	100	100	100	100
Collision Energy:	28.0	33.0	39.0	48.0	20.0	16.0
Collision Cell Entrance Potential:	10.0	10.0	10.0	10.0	7.00	7.00
Collision Cell Exit Potential:	20.0	20.0	16.0	21.0	20.0	20.0
Declustering Potential:	180	180	120	120	75.0	75.0

Other instrumentation may be used but may require optimization to achieve the desired separation and sensitivity. It is important to note that the parameters above have been established for this particular instrumentation and may not be applicable for other similar equipment that may be used.

2.12.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set. Calibration standards were interspersed among analysis of the recovery samples, approximately every two to six injections. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.13 Evaluation of Precision, Accuracy, Specificity, and Linearity

The accuracy was reported in terms of percent recovery of the fortified recovery samples. Recoveries of 70.0 to 110% (for the individual mean concentrations) are acceptable. The precision was reported in terms of the standard deviation (SD), overall relative standard deviation (RSD), and/or coefficient of variation (CV) for the recovery samples RSD values less than or equal to 20% were considered acceptable for the recovery samples. Specificity of

the method was determined by examination of the control samples for peaks at the same retention times as V-10456, S-3100-CA, and S-3100-CA-UR, which might interfere with the quantitation of the analytes. Linearity of the method was determined by the coefficient of determination (r^2), y-intercept, and slope of the regression line.

2.14 Limit of Quantitation (LOQ)

The method was validated at the LOQ. This was defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) did not exceed 30% of the LOQ.

2.15 Limit of Detection (LOD) and Method Detection Limit (MDL)

The LOD was calculated using three times the noise value of the control samples. Representative calculations for the LOD can be found in Section 3.0.

The MDL was defined as the lowest concentration in test samples which can be detected based on the concentration of the lowest calibration standard and the dilution factor of the control solutions. Representative calculations for the MDL can be found in Section 3.0.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) of the calibration standards against the peak area of the analyte in the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1)\quad y = mx + b$$

$$(2)\quad DC\,(x) = \frac{(y - b)}{m}$$

$$(3)\quad A = DC \times DF$$

where:

x	=	analyte concentration
y	=	detector response (peak area) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (µg/L), concentration in the original sample

The method detection limit (MDL) is defined as the lowest concentration that can be detected by this method in test solution samples. The MDL is calculated (Equation 4) based on the concentration of the lowest calibration standard and the dilution factor of the control samples:

$$(4)\quad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL}	=	lowest concentration calibration standard (e.g., 0.00500 µg/L for V-10456 and 0.0250 µg/L for S-3100-CA and S-3100-CA-UR)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
MDL	=	method detection limit reported for the analysis (e.g., 0.00500 µg/L × 10.0 = 0.0500 µg/L for V-10456; 0.0250 µg/L × 10.0 = 0.250 µg/L for S-3100-CA and S-3100-CA-UR)

The LOD was calculated using the following equation:

$$(5)\quad LOD = (3 \times (N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two lowest calibration standards
$Conc_{LS}$	=	concentration of the lowest calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
LOD	=	limit of detection for the analysis

5.0 DISCUSSION

The results described above, and those presented in Table 1 through Table 9, demonstrate that the method validated for V-10456, S-3100-CA, and S-3100-CA-UR that is to be used for testing met the established SANCO/3029/99 rev. 4 (EC, 2000), OCSPP 850.6100 (U.S. EPA, 2012), and protocol requirements for accuracy, precision, specificity, linearity, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of identification. Accuracy requirements were met because the mean recoveries of the recovery samples fell within the acceptable range of 70.0 to 110%. Precision was demonstrated through acceptable relative standard deviation for sample recovery values (≤20%). Specificity was demonstrated through reagent blanks and controls having quantifiable residues <30% of the LOQ. The calibration curves yielded $r^2 \geq 0.990$; therefore, acceptable regression analysis criteria were met. Limit of detection and method detection limits were also appropriately determined through the calculations previously presented in Section 3.0. Confirmation of identification was established through the confirmatory analysis adhering to the aforementioned method specifications.

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SMITHERS
INNOVATE WITH CONFIDENCE

TEST PROTOCOL

Title: V-10456, S-3100-CA and S-3100-CA-UR - Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions

Data Requirement(s): OCSPP 860.1340 and SANCO 3029/99

Test Substance(s): Refer to Section 3.0

Reference Substance(s): Refer to Section 3.0

Study Sponsor: Valent USA
Address: Valent Technical Center
6560 Trinity Court
Dublin, CA 94568

Study Monitor: Edward Scollon
Email: edward.scollon@valent.com
Phone Number: 925-948-2965

Project Number: 201660

Testing Facility: Smithers
790 Main Street
Wareham, Massachusetts 02571

Study Director: Stephen R. Devellis

Smithers Study No.: 12709.6509

Test Concentrations: See Internal Table in Section 4.0

Proposed Experimental Dates
Start: April 2020
Termination: April 2020

22 APR 2020
Sponsor Approval **Date**

22 April 2020
Study Director Signature **Study Initiation Date**

Environmental Risk Sciences Division
www.smithers.com

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V-10456, S-3100-CA and S-3100-CA-UR - Validation of the Analytical Method for the Determination of the Test Substance in Aqueous Solutions

1.0 INTRODUCTION

The purpose of this protocol is to validate an analytical method used to determine the concentration of the test substance in aqueous solutions. The analytical method will be validated with regards to specificity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), method detection limit (MDL), and confirmation of identification.

2.0 JUSTIFICATION OF THE TEST SYSTEM

This study is conducted to support the registration of the test substance.

The study will be conducted under Good Laboratory Practices (GLP) regulations and principles as described in 40 CFR 160 and the OECD principles on GLP, and will follow guidance documents SANCO/3029/99 REV 4 (2000) and OCSPP 860.1340.

3.0 TEST SUBSTANCE

3.1 Test Substance

Upon arrival at Smithers, the test substance will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage containers inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

The test substance will be given unique sample ID numbers and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, SDS, safe handling procedures, and a verified expiration or reanalysis date.

3.1.1 V-10456

Test Substance: V-10456 TG
Synonym: S-3100
Purity: 96.8%
Expiry Date: 23 November 2020
Lot #: 17TK11S3G

3.1.2 V-10456

Reference Substance: V-10456
Purity: 99.4%
Expiry Date: 03 April 2021
Lot #: AS 2429b

3.1.3 S-3100-CA

Test Substance S-3100-CA
Purity: 98.6%
Expiry Date: 30 November 2022
Lot #: 19SC8673272

3.1.4 S-3100-CA-UR

Test Substance S-3100-CA-UR
Purity: 97.1%
Expiry Date: 05 November 2021
Lot #: AS 2434a

3.2 Test Matrix

3.2.1 Dilute, Natural, Filtered Seawater (FSW)

The validation matrix to be used in this study will be Dilute, Natural, Filtered Seawater (FSW). Natural, filtered seawater from the Cape Cod Canal will be diluted with on-site laboratory well water to a salinity of 20±3% and filtered through a series of polypropylene core filters as fine as 1-µm. Salinity and pH of each new batch of seawater will be measured and recorded.

3.3 Reagents

Highly pure reagents will be used throughout the study. The actual reagent grade will depend on the manufacturer's designations. Generally these reagents will have grades, such as high purity solvent, ACS grade, or Select. The reagents used are recorded along with test chemical information at the time of preparation.

4.0 VALIDATION DESIGN

The test design will consist of Dilute, Natural, Filtered Seawater (FSW) fortified with the test substance at the concentrations listed in the table below. The concentrations will span the range of test concentrations to be used in studies involving the test substance. The control matrix for the validation will be selected from the matrices that will be used in the studies. In this case, the matrix used will be FSW . The validation study levels (approximate concentrations) for the test substances can be found below:

V-10456

Sample Type	Concentration	# of Replicates
Reagent Blank	0.00 µg a.i./L	2
Control Matrix	0.00 µg a.i./L	5
Control Matrix fortified at the LOQ	0.100 µg a.i./L	5
Control Matrix fortified at the High level	1000 µg a.i./L	5

S-3100-CA and S-3100-CA-UR

Sample Type	Concentration	# of Replicates
Reagent Blank	0.00 µg a.i./L	2
Control Matrix	0.00 µg a.i./L	5
Control Matrix fortified at the LOQ	1.00 µg a.i./L	5
Control Matrix fortified at the High level	1000 µg a.i./L	5

4.1 Specificity

The specificity of the method will be determined by applying the method to the appropriate number of control matrix samples and a minimum of two reagent blanks for each matrix. Chromatograms will be obtained for the control samples and examined for peaks that might interfere with the quantitation of the analyte peak of interest. Peaks attributable to the test substance should be sufficiently resolved from any peaks found in the samples of control matrix to enable quantification.

4.2 Linearity

The analytical calibration will extend over a range appropriate to the lowest and highest nominal concentration of the analyte in relevant analytical solutions ± at least 20%. A minimum of five concentrations will be utilized in the determination of the calibration line. The calibration data will be subjected to a regression analysis. The equation of the calibration line and a regression parameter, e.g., the coefficient of determination (r^2), will be reported and a typical calibration plot submitted. The data should have a coefficient of determination (r^2) of not less than 0.990 or a correlation coefficient (r) of not less than 0.995. A linear regression analysis will be used for the quantitation of recovery samples.

4.3 Matrix Enhancement/Suppression

The effects of matrix enhancement or suppression will be evaluated through the assessment of solvent-based and matrix-matched calibration standards. In the event that signal enhancement or suppression is observed, the Sponsor will be notified.

4.4 Accuracy

The accuracy of the method will be reported as the mean recovery ± the relative standard deviation (see Section 4.5 below) for the test substance in the sample matrix. Recovery determinations will be made on representative samples prepared by fortification of the control matrix with a known quantity of the analyte. Recovery data will be reported for two fortification levels appropriate to the proposed LOQ and likely testing levels (see Section 4.6 below). The fortification range will encompass the LOQ and the predicted highest level to be used during testing (see table, Section 4.0 above). The samples described above will be prepared '*de novo*'. Mean recoveries of 70.0-110% for each fortification level will be considered acceptable. During subsequent testing, individual quality control sample recovery acceptability will be set at 70.0 - 120%.

4.5 Precision

The precision of the method will be reported as repeatability of recovery at each fortification level. The number of determinations made at each fortification level for each validation is outlined in the table located in Section 4.0 above. The precision will be calculated for the fortified samples in terms of the standard deviation (SD), overall relative standard deviation (RSD), and/or coefficient of variation (CV). RSD values ≤ 20% for each fortification level will be considered acceptable. In certain justified cases higher variability may be accepted. RSD values outside of the ranges detailed above will be discussed with the Sponsor prior to proceeding.

4.6 Limit of Quantitation (LOQ)

The method will be validated at the limit of quantitation (LOQ). This will be defined as the lowest fortification level. Blank values (reagent blanks and untreated control samples) should not exceed 30% of the LOQ. If this is exceeded, it will be discussed with the Sponsor and detailed justification provided prior to proceeding.

4.7 Limit of Detection (LOD) and Method Detection Limit (MDL)

The limit of detection (LOD) will be calculated using three times the noise value of the control samples.

The method detection limit (MDL) will be set at the lowest concentration that can be detected in test solution samples. This value is calculated based on the concentration of the low calibration standard and the dilution factor of the control samples.

4.8 Confirmation of Analyte Identification

A chromatographic confirmatory method will be used to determine test solution concentrations during validation. Utilizing an LC-MS/MS system, the primary product ion and one confirmatory (secondary) product ion will be used for identification/quantification. The confirmatory ion analysis will also adhere to the aforementioned method specifications (Sections 4.1 – 4.7 above).

5.0 PROCEDURE FOR THE IDENTIFICATION OF THE TEST SYSTEM

The test system will be defined as the fortified recovery samples. The fortified recovery samples will be labeled as defined in Section 4.0 and each sample replicate will be assigned a unique identifier. Processing of fortified recovery samples will be performed at a lab station labeled with the study number.

6.0 CONTROL OF BIAS

Bias will be effectively controlled through techniques such as, but not limited to, preparation of replicate samples and replicate analysis.

7.0 SAMPLE DISPOSAL

All study specimens, and/or samples collected during the study, and test materials and reference standards, etc., provided by the sponsor, client, or customer will either be returned to the originator, shipped to a third party archival facility on behalf of the study sponsor who will incur the costs of shipping and archival, or disposed of according to Smithers SOPs.

8.0 RECORDS TO BE MAINTAINED

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

9.0 REPORTING

The raw data generated at Smithers will be peer-reviewed and the final report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process. The Quality Assurance Unit will inspect the final report to confirm that the methods, procedures, and observations are accurately and completely described, that the reported results accurately and completely reflect the raw data generated at Smithers and to confirm adherence with the study protocol. A single copy of the draft report will be submitted to the Study Sponsor for review. The report will be finalized according to Smithers standard operating procedures. The final report will meet the formatting requirements of EPA's PR Notice 2011-3. The report will include, but not be limited to, the following information:

- The report and project numbers from Smithers and Sponsor Study number.
- Laboratory and site, dates of testing and personnel involved in the study, i.e., Program Coordinator (if applicable), Study Director and Principal Investigator.
- Identification of the test substance including chemical name, additional designations (e.g., trade name), chemical designation (CAS number), empirical formula, molecular structure, manufacturer, lot or batch number, degree of purity of test substance (percent test chemical) (Sponsor supplied, if available).

- A full description of the experimental design and procedures followed and a description of the test equipment used.
- The determined specificity, linearity, accuracy, precision, LOQ, LOD and MDL, and confirmation of identification.
- The mathematical equations and statistical methods used in generating and analyzing the data as well as calculations using these equations. Tabular and graphical representations (if appropriate) of the data.
- Description of any problems experienced and how they were resolved.
- Good Laboratory Practice (GLP) Compliance Statement signed by the Study Director.
- Statement of non-confidentiality and that the method contains no trade secrets of proprietary data.
- Date(s) of Quality Assurance reviews, and dates reported to the Study Director and management, signed by the Quality Assurance Unit.

10.0 PROTOCOL CHANGES

All amendments to the approved protocol must be documented in writing and signed by both the Study Director and the Sponsor contact or representative. Protocol amendments and deviations must include the reasons for the change and the predicted impact of the change on the results of the study, if any. All changes to the protocol will be indicated in the final report.

11.0 GOOD LABORATORY PRACTICES

All test procedures, documentation, records and reports will comply with the U.S. Environmental Protection Agency's Good Laboratory Practices as set forth under the Federal Insecticide, Fungicide and Rodenticide Act (U.S. EPA, 1989) and as accepted by OECD Principles of Good Laboratory Practice (OECD, 1998).

12.0 REFERENCES

European Commission, 2000. Residues: Guidance for the generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part V, section 4) and Annex III (part A, section 5) of Directive 91/414. SANCO/3029/99 rev.4.

OECD, 1998. OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. Number 1. OECD Principles on Good Laboratory Practice (as revised in 1997). Environment Directorate Chemicals Group and Management Committee. ENV/MC/CHEM (98)17. OECD Paris. France. 41 pp.

U.S. EPA, 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule (40 CFR, Part 160); FR: 8/17/89; pp. 34052. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA, 1997. Office of Waste. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods (SW-846). Update IV, January 2008. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA, 2011. Pesticide Registration (PR) Notice 2011-3 Standard Format for Data Submitted Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and Certain Provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA). US Environmental Protection Agency Office of Pesticide Programs. November 30, 2011.

U.S. EPA. 1996. Office of Chemical Safety and Pollution Prevention. Residue Chemistry Test Guidelines, OCSPP 860.1340 Residue Analytical Method. EPA 712-C-96-174. August 1996. U.S. Environmental Protection Agency, Washington, D.C.