1.0 EXECUTIVE SUMMARY

North Coast Laboratories, Ltd. (NCL) performed an independent laboratory validation (ILV) Independent Laboratory Validation of the Analytical Method from the PTRL Europe ID: P 2376 G [1] as requested by Valent U.S.A Corporation. This study was designed to fulfil the requirements of EPA's Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2].

The purchased spring water samples were analyzed by direct injection using LC-MS/MS. A Applied Biosystems/MDS Sciex API 4000™ mass spectrometer with an ACE 5 C18 analytical column was used for the analysis. The instrument was calibrated using The Analyst® Software (version 1.6).

The analytical validation set consisted of one reagent blank, duplicate untreated control water samples (UTC), five UTC water samples fortified at 0.10 µg/L (the LOQ of the method) and five UTC water samples fortified at 1.0 µg/L (ten times the LOQ of the method).

The method was successfully validated on the first analysis of the spring water. The mean recoveries were within the acceptance range of 70 to 120% of the known quantity of reference standard spiked into the matrix blanks. The relative standard deviation (RSD) of replicate measurements was less than 10% for S-2200 at each spiking level. The analysis of the calibration standards associated with for S-2200 resulted in a standard curve with a correlation coefficient (r) greater than or equal to 0.995. The analysis of control matrix showed no interference with the peak area of analyte.

2.0 INTRODUCTION

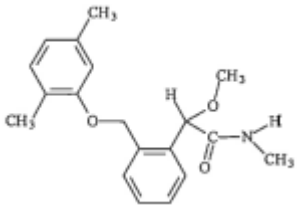
This report describes the independent laboratory validation (ILV) of of the Analytical Method from the PTRL Europe ID: P 2376 G [1] as performed by North Coast Laboratories, Ltd. (NCL) for the determination of the S-2200 in spring water using LC-MS/MS.

This study fulfils the requirements of EPA's Ecological Effects Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation [2].

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance was shipped from Valent U.S.A. Corporation, Dublin, CA to NCL. The S-2200 was received on January 9, 2013 and re-certified on May 9, 2013. The test substance that was used for the validation is described as follows:

Chemical Name	(*RS*) 2-[2,5-dimethylphenoxy)methyl]-α-methoxy-*N*-methyl-benzeneacetamide
Company Experimental Name	S-2200
CAS Name/Number	Not assigned
Source	Valent Technical Center
Purity	99.6% (wt/wt)
Lot Number	AS 2261c
Other Identification	(V-10190)
Expiration Date	May 9, 2015
Structural Formula	

Valent U.S.A. Corporation, Dublin, CA maintains the characterization and stability data for the test substance.

Stock standard solutions were prepared from the neat test substance for use in the preparation of fortification solutions and instrument calibration solutions. All standard solutions were prepared as per the method except at different concentrations. The stock standard was stored refrigerated when not in use. Section 3.5.4 describes of the preparation of the stock solutions, and Section 4.7.2 provides example calculations.

3.2 Equipment and Reagents

3.2.1 Solvents and Reagents

HPLC Water (Fisher)
Acetonitrile (EMD OmniSolv)
Formic acid (EMD)

3.2.2 Apparatus

A list of apparatus used in the method validation trial is shown below. Similar equipment from other suppliers may also be used.

Mettler AB204-2 Analytical Balance
10 mL volumetric flasks
Assorted syringes
Screw Thread Amber 15-mL glass vials with Teflon-lined screw-caps
CRS 1.8-mL clear screw top standard mouth glass autosampler vials with caps

3.2.3 LC-MS/MS Instrumentation

Analysis was performed using a liquid chromatograph with a mass specific detector (LC-MS/MS). The following equipment was used:

LC/MS-MS system-

Shimadzu LC-10 AD vp, isocratic pumps (two each)

Shimadzu SLC-10 A vp, pump controller (one each)

Perkin Elmer Series 200 autosampler

Phenomenex six-port, two position, switching valve for diverting column effluent to waste.

Applied Biosystems/MDS Sciex API 4000™ mass spectrometer with Turbo V® pneumatically assisted electrospray ionization interface

ACE 5 C18 50x3 mm id, p/n ACE-121-0503, batch V07-1307 with Phenomenex C-12 4x20 mm Guard column p/n AJ0-6073

3.3 Safety and Health

This method was performed by trained personnel who acted in accordance with the material safety data sheet (Appendix 4) that documents the hazards associated with the use of this chemical.

3.4 Test System and Sample Storage

South Fork Mountain Spring Water (bottled water) was used as the matrix for the validation. The water sample received a unique North Coast Laboratories, Ltd. (NCL) sample number. The sample was stored refrigerated after it had been opened. The sample was assigned a Sample Numeric ID of 1309200-01A.

3.5 Analytical Method and Method Establishment

3.5.1 Principle of the Method

The analysis was a direct injection of the spring water sample for analysis using LC-MS/MS.

3.5.2 Limits of Quantitation

The limit of quantitation (LOQ) for S-2200 was 0.10 µg/L (ppb). The analyst set the LOD to be half of the LOQ (0.05 µg/L).

3.5.3 Validation Sample Set

The validation set consisted of the following samples:

Six instrument calibration standards (0.50 – 2.0 µg/L)
One reagent blank (Fisher HPLC water)
Two unfortified control samples (Bottled spring water)
Five samples fortified with S-2200 at 0.10 µg/L (ppb; 1xLOQ)
Five samples fortified with S-2200 at 1.0 µg/L (ppb; 10xLOQ)

3.5.4 Preparation of 1000 PPM S-2200 Standard Solution

Section 4.7.1 provides an example calculation describing the preparation of the 1000-ppm stock standard solution.

An aliquot of 0.0102 g of analyte was weighed out into an amber glass vial. The appropriate amount of acetonitrile was added to the vial to yield a 1000 µg/mL standard solution. All concentrations of the S-2200 standard solutions were stored refrigerated at 2 to 6 ºC.

3.5.5 Preparation of S-2200 Fortification and Calibration Standard Solutions

A 10 µg/mL standard solution was prepared by adding 0.10 mL of the 1000 µg/mL standard to a 10-mL volumetric flask and bringing the solution up to the 10-mL final volume with acetonitrile. From this 10 µg/mL standard, a 0.10 µg/mL standard solution was prepared by combining 0.10 mL of the 10 µg/mL standard into a 10-mL volumetric flask and bringing the solution up to the 10-mL final volume with acetonitrile. From this 0.10 µg/mL standard, a 0.010 µg/mL standard solution was prepared by combining 1.0 mL of the 0.10 µg/mL standard into a 10-mL volumetric flask and bringing the solution up to the 10-mL final volume with acetonitrile. From this 0.10 µg/mL standard, a 0.0010 µg/mL standard solution was prepared by combining 1.0 mL of the 0.010 µg/mL standard into a 10-mL volumetric flask and bringing the solution up to the 10-mL final volume with acetonitrile.

3.5.6 Preparation of S-2200 Instrument Calibration Working Standard Solutions

Six levels of instrument calibration working standards were prepared (0.5x, 1x, 2x, 5x, 10x and 20x LOQ) and named with respect to the concentration in the fortified samples (see the table below and the example calculations presented in Section 4.7.3). The standards described in the table below were brought up to a final 1.0-mL volume with acetonitrile.

S-2200 Instrument Calibration Working Standard Solutions			
In solution Concentration and S-2200 Concentration Relative to the Sample	Concentration S-2200 Stock Solution (ng/µL)	Volume of S-2200 Stock Solution (µL)	Final Volume (mL)
0.5xLOQ = 0.05 µg/L	0.010	50	10
1xLOQ = 0.10 µg/L	0.010	100	10
2xLOQ = 0.20 µg/L	0.010	200	10
5xLOQ = 0.50 µg/L	0.100	50	10
10xLOQ = 1.0 µg/L	0.100	100	10
20xLOQ = 2.0 µg/L	0.100	200	10

3.5.7 Preparation of Samples

The control water sample used in this ILV was South Fork Mountain Spring Water purchased from a local grocery store. The only preparation involved dispensing a measured volume of the water into an autosampler vial, fortifying the water with either fortification solution or acetonitrile (method blanks), capping and mixing.

3.5.8 Preparation of Fortification Samples

A 100-µL aliquot of the 0.0010 ng/µL, (ppm) standard solution was added to each replicate LOQ fortification. A 100-µL aliquot of the 0.010 ng/µL, (ppm) standard solution was added to each replicate 10xLOQ fortification. Section 4.7.2 presents the calculations used to prepare the fortified samples.

3.5.9 Analysis Procedure

The analysis procedure was performed as described in the method in the PTRL Europe ID: P 2376G for the determining residues of S-2200 in surface. An excerpt of the report containing the method is incorporated into the Study Protocol which is presented in Appendix 2.

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3.5.10 LC-MS/MS Operating Parameters

3.5.10.1 Conditions

Column: ACE 5 C18 50x3 mm id, p/n ACE-121-0503, batch V07-1307 with Phenomenex C-12 4x20 mm Guard column p/n AJ0-6073

Injection volume: 20 µL
Flow Rate: 0.50 mL/min
Mobile Phase A: 0.1% formic acid in HPLC water
Mobile Phase B: 0.1% formic acid in acetonitrile
Gradient:

Time: 0.0	%A = 90	%B = 10
Time: 2.4	%A = 5	%B = 95
Time: 4.5	%A = 5	%B = 95
Time: 4.6	%A = 90	%B = 10

Expected Retention time: S-2200 2.9 min
Quantifier m/z: 314.2/192.0
Qualifier m/z: 314.0/160.0

Copies of example chromatograms are included in the Figures Section and the operating parameters for the LC-MS/MS are included in Appendix 3.

3.5.10.2 Calibration Procedures

Instrument calibration working standard solutions were prepared as described in Section 3.5.6. Six instrument calibration working standards were positioned within the analytical batch sequence, bracketing no more than three samples between standards. The standard concentrations were 0.5x, 1x, 2x, 5x, 10x and 20xLOQ (0.05, 0.10, 0.20, 0.50, 1.0, and 2.0 µg/mL, ppb, respectively). The Analyst® Software (version 1.6) generated a linear (with 1/x weighting) calibration curve and the associated correlation coefficient (r) for S-2200 by plotting the analyte peak area count versus analyte concentration. The correlation coefficient (r) was required to be greater than or equal to 0.995. The equation generated by Analyst® Software (version 1.6) was verified using Microsoft® Excel.

3.5.11 Data Acquisition and Reporting

The analysis of samples by LC-MS/MS generated electronic data via the Analyst® Software (version 1.6) interface. The hardware, security, and report configurations were set through the software modules. These modules also enabled instrument tuning, provided a mechanism for setting the acquisition methods and batches, processed the data, and quantified the data.

The Analyst Software generated raw data files from which the data were tabulated, and the chromatograms and the standard curves were generated. The data and the resulting descriptive statistics are summarized in Table 1 (Tables Section). Representative chromatograms are presented in the Figures Section.

3.5.12 Qualifier Ions

Data were collected (but not reported) for the qualifier ion 314.0/160.0 m/z.

4.5 Potential Problems, Hazards, or Precautions

There are no potential problems, hazards or precautions to report.

4.6 Communication with the Study Sponsor

All correspondence with the Sponsor were kept in a "Correspondence File".

Method changes and clarifications:
Prior to starting the ILV it was verified the following changes could be made:

- A linear calibration with 1/x weighing was used
- A different analytical column was to be used.
- Clarification of type and source of water to use.

Communication did not compromise the independent evaluation.

4.7 Calculations

4.7.1 Calculation of the Amount of Solvent Needed to Bring a Weighed Amount of Standard to the Required Concentration

$$\text{Volume of solvent} = \frac{(\text{weight of standard}) \times (\text{percent purity})}{\text{desired concentration}}$$

Example calculation
Preparation of the 1000 µg/mL (ng/µL, ppm) analyte standard stock solution:

Weight of analyte: 0.0102 g
Percent purity: 99.6%

Weight of analyte corrected for percent purity = (0.0102 g) x (996000 µg/g)
= 10159 µg

Volume of solvent needed = (10159 µg) / 1000 µg/mL
= 10.159 mL

4.7.2 Calculations used in the Preparation of Fortification Samples

LOQ Fortification

An LOQ fortification on a 1.0 mL water sample was prepared by fortifying the sample with 100-µL aliquot of the 0.0010 ng/µL, (ppm) fortification standard solution:

LOQ fortification = (100 µL) x (0.0010 ng/µL) x (1/1.1 mL) (1.1)* = 0.10 ng/mL (ppb)

10xLOQ Fortification

A 10xLOQ fortification on a 1.0 mL water sample was prepared by fortifying the sample with 100-µL aliquot of the 0.010 ng/µL, (ppm) fortification standard solution:

10 LOQ fortification = (100 µL) x (0.010 ng/µL) x (1/1.1 mL) (1.1) * = 1.0 ng/mL (ppb)

*The value of 1.1 represents the dilution factor due to the dilution from adding the 100 uL fortification to the 1.0 mL of sample.

4.7.3 Calculations Used in the Preparation of a 1xLOQ, 1 µg/L (ppb), Instrument Calibration Working Standard

In-solution concentration (calibration standard):

In-solution concentration (calibration standard) = (volume of stock standard) x (concentration of stock standard) x (1/final volume)

In-solution concentration (calibration standard) = (100 µL) x (0.010 ng/µL) x (1/10 mL)
= 0.10 ng/mL (ppb)

This agrees with the analyte concentration in a final sample volume of 1.1 mL (with a dilution factor 1.1) at 100% recovery (Section 4.7.2). Instrument calibration working standards at other concentrations were calculated in a similar manner as listed in Section 3.5.6.

4.7.4 Calculation of Analyte Concentrations

The Analyst® Software (version 1.6) generated regression equations with a linear fit and a 1/x weighting factor from the concentration of each standard and its peak area count. The reported data in Table 1 employed a linear 1/x weighting factor. The regression equation was used to calculate the concentration of each sample by applying the respective peak area count to the curve. The calculated concentration that is recorded on each chromatogram in the

Figures Section, and reported in Table 1, is expressed in terms of µg/L (PPB). The equation generated by Analyst® Software (version 1.6) for the curve was verified using Microsoft® Excel. The calibration curve and regression equation generated by Analyst® Software (version 1.6) are shown in Figure 1.

The calibration equation is calculated as $Y = mX + b$
Rearranging:

$X = (Y - b) / m$

Y = analyte peak area count
X = analyte concentration (µg/L)
m = slope
b = y-intercept

Example proof of calculation (see Figure 1 for regression equation) for the LOQ analyte fortification #1 (Figure 10), where the coefficients were calculated by Microsoft® Excel to show increased significant figures where:

Y = 41160
m = 404593
b = 4675
X = unknown
DF (dilution factor) = 1.1

$X = ((Y - b) / m) DF$

$X = ((41160 - 4675) / 404593) 1.1$

X = 0.09920 µg/L

NOTE: The regression coefficients used by the by the Analyst® Software (version 1.6) software to calculate residues include more significant figures than the rounded numbers printed out in the data packages. Therefore, the software calculation cannot always be reproduced exactly beyond the third/fourth significant figure by Microsoft® Excel or a hand-held calculator.

4.7.5 Calculation of Method Fortification Percent Recovery

$$\text{Method fortification recovery (\%)} = \frac{\text{residue found (µg/L)}}{\text{target fortification concentration (µg/L)}} \times 100\%$$

The LOQ S-2200 fortification #1, 0.10 µg/L (ppb), (see Table 1, and Figure 10)

Method fortification recovery (%) = (0.09920 µg/L / 0.1 µg/L) x 100% = 99.2%

4.7.6 Calculation of Standard Deviation (s) and Relative Standard Deviation (RSD)

Standard deviations for the mean percent recoveries in Table 1 were calculated in a Microsoft® Excel spreadsheet and verified using a calculator. The standard deviation is designated as "s" and the percentage is expressed as an absolute number.

Relative standard deviation (RSD)

Relative standard deviation = (standard deviation / mean) x 100%

Example calculation
The five replicates of the LOQ analyte fortification presented in Table 1 resulted in an unrounded mean recovery of 96.912% and an unrounded standard deviation of 1.554854%. The RSD was calculated as:

RSD = (1.554854% / 96.912%) x 100%

= 1.604%

= 1.6%

4.7.7 Calculation of 95% Confidence Interval for a Mean

$$95\%\ \text{confidence interval} = \text{mean} \pm \frac{t \times \text{standard deviation}}{\sqrt{n}}$$

Where: n = number of measurements
t = student t variate for n-1 degrees of freedom at the 95% confidence interval (2.776 for 4 degrees of freedom) [4].

Example calculation
From the five recovery values of water samples fortified with analyte at LOQ level (Table 1), the unrounded mean recovery was 96.912% and the unrounded standard deviation was 1.554854%. The confidence interval was calculated as:

$$95\%\ \text{confidence interval} = 96.91\% \pm \frac{(2.776)\,(1.554854\%)}{\sqrt{5}}$$

$$= 96.91\% \pm 1.9303\%$$

$$= 96.91\% \pm 1.9\%$$

4.8 Statistics Statement

The mean percent recoveries were arithmetic means, and standard deviations for the mean percent recoveries were calculated and designated as "s" with the percentage expressed as an absolute number. Analyst® Software (version 1.6) calculated the regression coefficient and the correlation coefficient (r) associated with the linear standard calibration. Analyst®

Software (version 1.6) calculated the concentration of each sample by applying the peak area count of each sample to the calibration curve.

The individual recoveries of all fortifications were between 70 and 120%. The correlation coefficient (r) for each standard curve was greater than or equal to 0.995. Mean percent recoveries, standard deviations, relative standard deviations, and 95% confidence intervals were calculated in a Microsoft® Excel spreadsheet and verified with a calculator.

4.9 Protocol, Method, or SOP Deviations

One SOP Deviation was prepared and is at the end of Appendix 3. The SOP deviation was written because an entry was not completed for a refrigerator key log.

5.0 CONCLUSION

The Valent U.S.A. Corporation analytical method described in the PTRL Project PTRL Europe ID: P2376G for determining residues of S-2200 in surface water was used successfully to determine the magnitude of residues in a spring water sample fortified with S-2200. The LOQ of 0.10 µg/L (ppb) was validated. The mean recoveries, standard deviations, relative standard deviations and confidence intervals (95%) were within the regulatory guidelines [2], demonstrating the precision and accuracy of the method and successful validation of the method (see Method included within the Protocol, Appendix 2).

6.0 REFERENCES

1. Nicole Wilde; Method PTRL Europe ID: P 2376G Validation of an Analytical Method for the Determination of S-2200 in Surface Water for Post-Registration Control and Monitoring Purpose; October 19, 2011.
2. U.S. Environmental Protection Agency, Ecological Effects Test Guidelines OCSPP-850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.
3. U.S. Environmental Protection Agency, Office of Compliance Monitoring. 1989. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards; Final Rule, 40 CFR, Part 160. Federal Register, Vol. 54, No. 158: pp. 34052-34074.
4. John Keenan Taylor, Quality Assurance of Chemical Measurements, Lewis Publishers, Inc., 1987; Appendix C, Table C.3, page 267.