SUMMARY

The purpose of this study was to conduct an independent laboratory validation on ABC Laboratories, Inc. method study number 81340 entitled "Method Validation for Niclosamide in Ecotoxicology Media" (Leak. 2015). This ILV study was required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA. 2012) to confirm that the original analytical method, developed by one laboratory, can be independently validated by a second laboratory with no major interaction between the two laboratories. The method was successfully validated on the first attempt in freshwater and 20X Algal Assay Procedure (AAP) medium (a freshwater algal medium) at the method LOQ (0.0200 mg/L) and 10X LOQ (0.200 mg/L) concentration levels and in artificial sediment at concentrations of 0.200 and 2.00 mg/kg, respectively, using the method as written. The method was successfully confirmed using an alternative column to conduct the high performance liquid chromatographic (HPLC-UV) analysis.

1.0 INTRODUCTION

Methodology provided by Great Lakes Fishery Commission (ABC study number 81340, Leak. 2015) was validated to quantify the concentration of niclosamide present in recovery samples prepared in freshwater, 20X Algal Assay Procedure (AAP) medium (a freshwater algal medium) and artificial sediment on 11 through 21 June 2015. This independent laboratory validation (ILV) study is required by U.S. EPA under Guideline No. 850.6100 (U.S. EPA, 2012) to confirm that the original analytical method, developed by one group, can be independently validated by a second group with no major interaction between the two groups. This method was validated by fortification of freshwater and 20X AAP with niclosamide at concentrations of 0.0200 mg/L (LOQ) and 0.200 mg/L (10X LOQ) and fortification of artificial sediment at concentrations of 0.200 mg/kg (LOQ) and 2.00 mg/kg (10X LOQ). Freshwater and 20X AAP recovery samples were diluted with 100% methanol and the high-level recovery samples were further diluted into the calibration standard range with 20:80 methanol:purified reagent water (v:v). Artificial sediment recovery samples were extracted three times with 100% methanol and concentrated under a gentle stream of nitrogen at room temperature. The concentrated extracts were diluted with purified reagent water to a final composition of 20:80 methanol:purified reagent water (v:v) and the high-level recovery samples were further diluted into the calibration standard range with 20:80 methanol:purified reagent water (v:v). Samples were analyzed using high performance liquid chromatography (HPLC-UV). Samples were additionally analyzed using an alternative column to serve as a confirmatory method.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled "Niclosamide - Independent Laboratory Validation of the Analytical Method for Determination of Niclosamide in Water and Sediment by HPLC-UV" (Appendix 1). The methods described in this protocol meet the requirements specified in OCSPP Guidelines 850.6100 for Environmental Chemistry Methods and Associated Independent Laboratory Validation (U.S. EPA. 2012), 860.1340 for Residue Analytical Method (U.S. EPA. 1996a) and 850.7100 for Data Reporting for Environmental Chemistry Methods (U.S. EPA. 1996b).

2.2 Test Systems

The test systems used in this study were freshwater, 20X Algal Assay Procedure (AAP) medium and artificial sediment. Freshwater used in the study was laboratory well water reconstituted for hardness and was prepared in 1900-L batches by fortifying well water according to the formula for hard water (U.S. EPA. 1975) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. 20X AAP is a nutrient rich medium utilized in ecotoxicology studies. All documentation relating to the preparation, storage and handling is maintained by Smithers Viscient. The artificial sediment used for the study was artificial sediment (lot #111814) and was prepared by combining sphagnum peat moss, kaolin clay, and fine silica sand at appropriate amounts of 5, 20 and 75% (dry weight), respectively, in order to achieve a total percent organic carbon of 2 %. All documentation relating to the preparation, storage and handling is maintained by Smithers Viscient. Artificial sediment moisture content was determined prior to testing using a Sartorius moisture analyzer Model MA-45 and was determined to be 33.86%.

2.3 Test Substance

The test substance, niclosamide, was received on 27 May 2015 from Sigma-Aldrich Incorporated, Milwaukee, Wisconsin. The following information was provided:

Name:	niclosamide
Batch No.:	BCBN1110V
CAS No.:	50-65-7
Purity:	100.0%
Expiration Date:	27 May 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7696) was stored at room temperature in a dark, ventilated cabinet in the original container. Concentrations were adjusted for the purity of the test substance.

2.4 Reagents

1.	Methanol:	EMD, reagent grade
2.	Purified reagent water:	prepared from a Millipore Milli-Q® Direct 8 system (meeting ASTM Type II requirements)
3.	Acetic acid:	EMD, reagent grade
4.	Sodium acetate anhydrous:	Omnipur, reagent grade

2.5 Equipment

1.	Instrument:	Agilent Infinity Series 1260 ALS autosampler equipped with an Agilent Infinity Series 1260 quaternary pump, an Agilent Infinity Series 1260 DAD (diode array detector), an Agilent Infinity Series 1260 thermostatted column compartment and Agilent ChemStation ECM Version B.04.03 for data acquisition
2.	Balance:	Mettler Toledo XSE205DU, Mettler Toledo AG285, Mettler PJ-3000
3.	Moisture balance:	Mettler Toledo HB43-S
4.	Shaker table:	VWR 3500
5.	Centrifuge:	Beckman Allegra X-12

6.	Ultra-centrifuge:	Eppendorf 5417C
7.	Laboratory equipment:	volumetric flasks, disposable glass pipets, disposable glass vials, positive displacement pipets, Nalgene® centrifuge tubes, graduated plastic centrifuge tubes, autosampler vials and amber glass bottles with Teflon®-lined caps

2.6 Preparation of Reagents

A 20:80 methanol:purified reagent water (v:v) liquid reagent solution was typically prepared by combining 100 mL of methanol and 400 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 58 mM acetate buffer, pH 5 in purified reagent water liquid reagent solution was typically prepared by combining 4.6733 g of sodium acetate anhydrous, approximately 130 drops of acetic acid (until pH 5 was achieved) and 1.00 L of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 15:85 58 mM acetate buffer in purified reagent water:methanol (v:v) mobile phase solution was typically prepared by adding 150 mL of 58 mM acetate buffer in purified reagent water to 850 mL of methanol. The solution was mixed well using a stir bar and stir plate for five minutes, then degassed under vacuum with sonication for ten minutes.

2.7 Preparation of Stock Solutions

A 1000 mg/L primary stock solution was prepared by bringing 0.0251 g of niclosamide to a final volume of 25.0 mL with methanol. Two secondary stock solutions (1.00 and 10.0 mg/L) were prepared by bringing 0.0500 and 0.500 mL, respectively, of the 1000 mg/L primary stock solution each to a final volume of 50.0 mL with methanol.

All primary and secondary stock solutions were stored refrigerated in amber glass bottles fitted with Teflon®-lined caps.

2.8 Preparation of Calibration Standards

Calibration standards were prepared in 20:80 methanol:purified reagent water (v:v) at concentrations of 0.00500, 0.0100, 0.0200, 0.0500, 0.100 and 0.200 mg/L by fortifying with the 1.00 and 10.0 mg/L secondary stock solutions.

2.9 Sample Fortification and Preparation

2.9.1 Freshwater and 20X AAP Samples

All recovery samples were individually prepared in disposable glass vials containing 8.00 mL of freshwater or 20X AAP at each concentration level by fortification with the appropriate stock solution. Five replicates were prepared at each concentration level in disposable glass vials as follows.

Matrix	Sample ID	Stock Concentration (mg/L)	Volume of Stock Solution (mL)	Final Volume (mL)	Fortified Sample Concentration (mg/L)
Freshwater	Reagent Blank-1[a]	NA[b]	NA	8.00	0.00
	Control A & B	NA	NA	8.00	0.00
	LOQ A, B, C, D & E	1.00	0.160	8.00	0.0200
	High A, B, C, D & E	10.0	0.160	8.00	0.200
20X AAP	Reagent Blank-2[a]	NA	NA	8.00	0.00
	Control C & D	NA	NA	8.00	0.00
	LOQ F, G, H, I & J	1.00	0.160	8.00	0.0200
	High F, G, H, I & J	10.0	0.160	8.00	0.200

[a] 100% purified reagent water used as matrix in reagent blank.
[b] NA = Not Applicable.

Two additional 8.00 mL samples were prepared in each matrix and left unfortified to serve as controls. An additional sample was prepared using only purified reagent water to serve as the reagent blank for the analysis of each matrix.

Samples were immediately diluted with 100% methanol to a final volume of 10.0 mL. The high-level recovery samples were further diluted into the calibration standard range with 20:80 methanol:purified reagent water (v:v). Samples were transferred to autosampler vials and analyzed by HPLC-UV. A typical dilution is described below.

Matrix	Sample ID	Nominal Concentration (mg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
Freshwater	Reagent Blank-1	0.00	8.00	10.0	NA[c]	NA	1.25
	Control A & B	0.00	8.00	10.0	NA	NA	1.25
	LOQ A, B, C, D & E	0.0200	8.00	10.0	NA	NA	1.25
	High A, B, C, D & E	0.200	8.00	10.0	1.00	10.0	12.5
20X AAP	Reagent Blank-2	0.00	8.00	10.0	NA	NA	1.25
	Control C & D	0.00	8.00	10.0	NA	NA	1.25
	LOQ F, G, H, I & J	0.0200	8.00	10.0	NA	NA	1.25
	High F, G, H, I & J	0.200	8.00	10.0	1.00	10.0	12.5

[a] Diluted with 100% methanol.
[b] Diluted with 20:80 methanol:purified reagent water (v:v).
[c] NA = Not Applicable.

2.9.2 Artificial Sediment Samples

A total of 12 artificial sediment recovery samples (1.00 g dry weight) were weighed into individual 50-mL Nalgene® centrifuge tubes and were fortified with the appropriate stock solutions at concentrations of 0.200 and 2.00 mg/kg (dry weight). Five replicates were prepared for each concentration level as follows.

Sample ID	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Sample Concentration (mg/kg)
Reagent Blank-3	NA[a]	NA	NA	0.00
Control E & F	NA	NA	1.00	0.00
LOQ K, L, M, N & O	1.00	0.200	1.00	0.200
High K, L, M, N & O	10.0	0.200	1.00	2.00

[a] NA = Not Applicable.

Two additional 1.00 g (dry weight) samples were prepared and left unfortified to serve as controls. An additional sample was extracted using only solvents to serve as the reagent blank (no test materials or matrix) for the analysis.

A 2.0-mL aliquot of 100% methanol was added to each artificial sediment recovery sample (1.00 g dry weight) and they were placed on a shaker table for 30 minutes at 150 rpm. Samples were then centrifuged at 3000 rpm for 10 minutes and the extracts were transferred to graduated centrifuge tubes. The extraction and centrifugation procedures were repeated two additional times with an additional 2.0-mL aliquot of 100% methanol each time. The extracts were combined and concentrated to a volume of 2.0 mL under a gentle stream of nitrogen at room temperature. An 8.0-mL aliquot of purified reagent water was added to each concentrated extract for a final composition of 20:80 methanol:purified reagent water (v:v). The high-level recovery sample extracts were further diluted into the calibration standard range with 20:80 methanol:purified reagent water (v:v). Samples were centrifuged at 13,000 rpm for five minutes and were transferred to autosampler vials and analyzed by HPLC-UV. The extraction and dilution procedures are detailed below.

Sample ID	Nominal Concentration (mg/kg)	Dry Weight (g)	Extract Volume[a] (mL)	Final Volume[b] (mL)	Sample Volume (mL)	Final Volume[c] (mL)	Dilution Factor
Reagent Blank-3	0.00	NA[d]	6.00	10.0	NA	NA	10.0
Control E & F	0.00	1.00	6.00	10.0	NA	NA	10.0
LOQ K, L, M, N & O	0.200	1.00	6.00	10.0	NA	NA	10.0
High K, L, M, N & O	2.00	1.00	6.00	10.0	1.00	10.0	100

[a] Exracted with 100% methanol.
[b] Diluted with purified reagent water.
[c] Diluted with 20:80 methanol:purified reagent water (v:v).
[d] NA = Not Applicable.

2.10 Analysis

2.10.1 Instrumental Conditions

The high performance liquid chromatographic (HPLC-UV) analysis was conducted utilizing the following instrumental conditions:

Primary column:	Waters Symmetry C18, 3.5 µm, 75 mm x 4.6 mm
Confirmatory Column:	Agilent Zorbax SB-C18, 3.5 µm, 75 mm x 4.6 mm
Mobile Phase (A):	15:85 58 mM acetate buffer in purified reagent water:methanol (v:v)
Isocratic:	100% mobile phase A
Run Time:	5.00 minutes
Flow Rate:	1.00 mL/minute
Injection Volume:	50.0 µL for aqueous and 200 µL for artificial sediment
Wavelength:	335 nm
Column temperature:	25 °C
Retention Time:	approximately 2.7 minutes aqueous and 2.9 minutes artificial sediment (primary column) approximately 2.2 minutes aqueous and 2.3 minutes artificial sediment (confirmatory column)

2.10.2 Preparation of Calibration Standard Curve

Two sets of calibration standards were analyzed with each sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of recovery samples and calibration standards onto the chromatographic system was performed by programmed automated injection.

2.10.3 Method Differences

The method validation (Leak. 2015) was followed as written with the following exceptions:

- The method validation indicates centrifuging the soil samples at 3400 rpm, however, this speed was not achievable with the available equipment. All samples were centrifuged at 3000 rpm.

- The method validation indicates an injection volume of 50 µL, however, during the analysis of artificial sediment samples, poor chromatography and results were obtained. Therefore, the injection volume was increased to 200 µL to obtain optimal chromatography.

2.11 Evaluation of Precision, Accuracy, Specificity and Linearity

The accuracy was reported in terms of percent recovery of the low- and high-level recovery samples. Recoveries of 70 to 120% of nominal were considered acceptable, with no corrections made for procedural recoveries during the study. The precision was reported in terms of the standard deviation and relative standard deviation (RSD) for the retention time, the peak area quantitation, and the percent recovery values of the low- and high-level recovery samples for each analyte. The retention time should have an RSD of less than or equal to 2%. The RSD of the peak area based quantitation and of the recovery values should be less than or equal to 20%. Specificity of the method was determined by examination of the control samples for peaks at the same retention time as niclosamide which might interfere with the quantitation of the analyte. Interferences with peak areas that are less than 30% at the limit of quantification (LOQ) are not considered significant. Linearity of the method was determined by the correlation coefficient (r), y-intercept and slope of the regression line. The signal response data should have an intercept close to zero and a correlation coefficient not less than 0.995 with the calculated calibration standard concentrations within 20% of the theoretical value. The precision of the method at the LOQ was reported in terms of the relative standard deviation or coefficient of variation of the observed recovery values.

2.13 Time Required for Analysis

A normal batch of samples consists of 10 fortified and 2 unfortified samples, 1 reagent blank and 6 solvent standards (19 samples total). A single analyst completed two batches of aqueous samples for a set of 32 samples in one working day (8 hours) with HPLC-UV analysis performed overnight, and one batch of artificial sediment samples for a set of 19 samples in one working day (8 hours) with HPLC-UV analysis performed overnight.

3.0 Calculations

A calibration curve was constructed by plotting the analyte concentration (mg/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) $y = mx + b$

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

(3) $A = DC \times DF$

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 (mg/L) in the sample
DF	=	dilution factor (final volume of the sample divided by the original sample volume)
A	=	analytical result (mg/L or mg/kg), concentration in the original sample

The limit of quantitation (LOQ) was calculated using the following equation:

$$(4)\ LOQ_{INST} = \frac{(A_{LS}) - b}{m}$$

$$(5)\ LOQ = LOQ_{INST} \times DF_{CNTL}$$

where:

A_{LS}	=	mean detector response (peak area) of the low concentration calibration standard (two injections)
b	=	y-intercept of the linear regression
m	=	slope of the linear regression
LOQ_{INST}	=	limit of quantitation of the instrument
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used)
LOQ	=	limit of quantitation reported for the analysis

The limit of detection (LOD) was defined as the lowest calibration standard used in the analysis.