SUMMARY

The purpose of this study was to conduct an independent laboratory validation on ABC Laboratories, Inc. method study number 81341 entitled "Method Validation for 3-(trifluoromethyl)-4-nitrophenol (TFM) in Ecotoxicology Media" (Lucash, 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 (0.0600 and 0.0200 mg/L, respectively) and 10X LOQ (0.600 and 0.200 mg/L, respectively) concentration levels, 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 81341, Lucash, 2015) was validated to quantify the concentration of TFM present in recovery samples prepared in freshwater and 20X Algal Assay Procedure (AAP) medium (a freshwater algal medium) on 12 through 15 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 TFM at concentrations of 0.0600 and 0.0200 mg/L (LOQ), respectively, and 0.600 and 0.200 mg/L (10X LOQ), respectively. All 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). Samples were analyzed using high performance liquid chromatography (HPLC/UV). Samples were additionally analyzed using an alternative column to serve as a confirmatory method.

The study was initiated on 4 June 2015, 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 ILV study was conducted on 12 through 15 June 2015 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data and the final report produced during this study are archived at Smithers Viscient at the above location.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

This study was performed following the Smithers Viscient protocol entitled "TFM - Independent Laboratory Validation of the Analytical Method for Determination of TFM in Water 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 and 20X Algal Assay Procedure (AAP) medium. 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.

2.3 Test Substance

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

Name:	3-(trifluoromethyl)-4-nitrophenol
Synonym:	TFM
Batch No.:	MKBQ2965V
CAS No.:	88-30-2
Purity:	99.8%
Expiration Date:	27 May 2016

Upon receipt at Smithers Viscient, the test substance (SMV No. 7697) 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
3.	Laboratory equipment:	volumetric flasks, disposable glass pipets, disposable glass vials, positive displacement pipets, 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 60.0 mL of methanol and 240 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 4.7633 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 using a stir bar and stir plate for five minutes.

A 30:70 58 mM acetate buffer in purified reagent water:methanol (v:v) mobile phase solution was typically prepared by adding 300 mL of 58 mM acetate buffer in purified reagent water to

700 mL of methanol. The solution was mixed using a stir bar and stir plate for five minutes, followed by degassing under vacuum with sonication.

2.7 Preparation of Stock Solutions

A 1000 mg/L primary stock solution was prepared by bringing 0.0251 g of TFM (0.0250 g as active ingredient) to a final volume of 25.0 mL with methanol. Three secondary stock solutions (1.00, 10.0 and 100 mg/L) were prepared by bringing 0.0500, 0.500 and 5.00 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 glass amber 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

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	10.0	0.0480	8.00	0.0600
	High A, B, C, D & E	100	0.0480	8.00	0.600
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. One 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	0.0600	8.00	10.0	NA	NA	1.25
	High A, B, C, D & E	0.600	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.

2.10 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):	30:70 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
Wavelength:	295 nm
Column temperature:	25 °C
Retention Time:	approximately 2.4 minutes (primary column) approximately 2.1 minutes (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

There were no method differences between the method validation (Lucash, 2015) and this procedure.

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 TFM 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.

2.12 Communications

Communications occurred with the Sponsor Monitor to discuss items such as 1) clarification/approval of the protocol and method, 2) acquisition of analytical standard and 3) approval of final ILV results. A complete list of communications is maintained in the study raw data.

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 samples for a set of 32 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), 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.

REFERENCES

Lucash, K., 2015. Method Validation for 3-(trifluoromethyl)-4-nitrophenol (TFM) in Ecotoxicology Media. Document Number 81341.

U.S. EPA, 1975. *Methods for acute toxicity test with fish, macroinvertebrates and amphibians.* Ecological Research Series (EPA-660/3-75-009). U.S. Environmental Protection Agency, Washington, DC., 61 pp.

U.S. EPA, 1989. 40 CFR, Part 160. Federal Insecticide, Fungicide, and Rodenticide Act. Good Laboratory Practices Standards; Final Rule. Office of the Federal Register, National Archives and Records Administration. U.S. Government Printing Office, Washington, D.C.

U.S. EPA, 1996a. Office of Chemical Safety and Pollution Prevention. Ecological Effects Guideline, OCSPP 860.1340. Residue Analytical Methodology. EPA 712-C-96-174. August 1996. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA, 1996b. Office of Chemical Safety and Pollution Prevention. Ecological Effects Guideline, OCSPP 850.7100. Data Reporting for Environmental Chemistry Methods. EPA 712-C-96-348. August 1996. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA, 2012. Office of Chemical and Safety Pollution Prevention. Ecological Effects Test Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation. EPA 712-C-001. U.S. Environmental Protection Agency, Washington D.C.