1.0 ABSTRACT

Method Validation for 3-(trifluoromethyl)-4-nitrophenol (TFM) in Ecotoxicology Media

This report describes the validation of an analytical method for the analysis of 3-(trifluoromethyl)-4-nitrophenol (TFM) in various media that will be used during ecotoxicological testing. The method validation utilized a high-performance liquid chromatography system equipped with a UV detector and a Waters Symmetry C18 column. Quantitation was performed by external standard calibration using peak areas.

The method was shown to be valid for the analysis of TFM and TFM HP (Sea Lamprey Larvicide, treatment grade TFM) in the two ecotoxicology media (freshwater and 20X FWAM) tested. The accuracy, precision, recovery, and linearity data have shown this method to be acceptable for TFM and TFM-HP. Sample solution stability after at least seven day's refrigerator storage was acceptable for TFM and TFM-HP.

2.0 INTRODUCTION

The objective of this study was to validate the method for the analysis of 3-(trifluoromethyl)-4-nitrophenol (TFM) in various media that will be used during ecotoxicological testing. The study was conducted as described in the ABC study protocol titled "Method Validation for 3-(trifluoromethyl)-4-nitrophenol (TFM) in Ecotoxicology Media," which was patterned after the European Commission Working Document SANCO/3030/99 rev. 4 (1).

This report accurately reflects what was actually performed during the course of the study.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Test And Reference Substance

ABC Laboratories received the test substance 3-(trifluoromethyl)-4-nitrophenol (TFM) (Lot No.: MKBQ2306V) from Sigma-Aldrich on 06 June 2014. The test substance was given the ABC designation of MM-10333-00001 and was stored at room temperature. A non-GLP Certificate of Analysis was provided with the test substance. ABC Laboratories' subsequently determined the purity of the test substance to be 100% under GLP conditions (Appendix A), and a recertification date of 25 September 2016 was assigned.

ABC Laboratories received the TFM HP Sea Lamprey Larvicide (Lot No.: LAM140501A) from the United States Geological Survey on 24 September 2014. The test substance was given the ABC designation of MM-10947-00001 and was stored at room temperature. A non-GLP Certificate of Analysis was provided with the test substance. ABC Laboratories' subsequently determined the purity of the test substance to be 32.9% TFM under GLP conditions (Appendix A), and a recertification date of 15 October 2016 was assigned.

3.1.2 Test Systems

3.1.2.1 Freshwater Test Media

Freshwater was prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis (RO). The well water and RO water were blended together to yield a total hardness of 130 to 160 mg/L as $CaCO_3$. Characterization of the base water, i.e., ABC well water, used to prepare the dilution water can be found in Appendix B.

3.1.2.2 20X Freshwater Algal Nutrient Medium (20XAAP)

The 20XAAP medium (2) was prepared by the addition of appropriate reagent grade salts to autoclaved ABC reagent water. ABC reagent water is produced by passing reverse-osmosis water through a series of deionization tanks, a laboratory water purification system consisting of carbon, de-mineralization, and organic adsorption cartridges, and then through a 0.2-µm filter. After preparation, the medium was adjusted to pH 7.5 ± 0.1 with 0.1N HCl and filtered through

Millipore 0.22-µm filters. Chemical characterization of a representative sample of ABC well water, the base water used to prepare ABC reagent water, is presented in Appendix B.

3.1.3 Reagents

All reagents employed in this study were ACS reagent grade or purer.

3.1.4 Equipment

- Balance: Mettler XP205DR; Mettler MS 1003S
- pH meter: WTW Model pH 330i
- Sciex 4000 liquid chromatographic/mass spectrometry (LC-MS/MS) system
- Agilent HPLC-UV system
- HPLC column: Waters Symmetry C18
- Volumetric glassware
- Miscellaneous laboratory glassware

3.2 Methods

The study was conducted as described in the ABC protocol entitled, "Method Validation for 3-(trifluoromethyl)-4-nitrophenol (TFM) in Ecotoxicology Media" and amendments (Appendix C).

3.2.1 Preparation of Analytical Standard and Matrix Spiking Solutions

A primary stock solution of TFM was prepared on 22 August 2014 by dissolving 20.1 mg TFM in 50 mL of methanol, resulting in a concentration of 0.401 mg a.i./mL (after correcting for a purity of 99.8%). Subsequent dilutions of this primary stock solution in 20:80 methanol:water were used as analytical standards.

A second primary stock solution of TFM was prepared on 22 August 2014 by dissolving 59.6 mg TFM in 50 mL of methanol, resulting in a concentration of 1.19 mg a.i./mL (after correcting for a purity of 99.8%). A subsequent dilution in methanol was used as a fortification solution.

A third primary stock solution of TFM was prepared on 26 August 2014 by dissolving 502.1 mg TFM in 100 mL methanol, resulting in a concentration of 5.01 mg a.i./mL (after correcting for a purity of 99.8%). This primary stock solution was used as a fortification solution.

A primary stock solution of TFM-HP was prepared on 07 October 2014 by dissolving 151.3 mg TFM-HP in 50 mL of methanol, resulting in a concentration of 3.03 mg Total Product (T.P.)/mL (based on a purity of 100% formulation). Subsequent dilutions of this primary stock solution in methanol and 20:80 methanol:water were used as fortification solutions and certificate of analysis determination.

All solutions were stored refrigerated when not in use.

3.2.2 Sample Analysis

Water sample analysis was accomplished by dilution of samples with methanol and further dilution, if necessary, with 20:80 methanol:water, followed by direct analysis performed on a high-performance liquid chromatography system equipped with an ultraviolet detector (HPLC-UV).

3.2.3 Instrument Conditions

Instrument:	Agilent 1100 HPLC with ultraviolet detector
Column:	Waters Symmetry C18, 3.5 µm, 75 mm × 4.6 mm
Column Temp:	25 °C
Isocratic Mobile Phase:	30:70 58mM acetate buffer in water:methanol
Flow Rate:	1.0 mL/min
Injection Volume:	50 µL
Run Time:	5.0 minutes
Wavelength:	295 nm

Note: Instrument conditions may be changed to optimize chromatography.

3.2.4 Calculations

Calculation of TFM concentrations in test solution samples analyzed by HPLC-UV were performed by the external standard analysis function of Empower 2 and Empower 3 software. The concentration of the analyte in each sample was determined directly from the standard curve by the following equation:

$$\frac{(\text{Concentration from standard curve in mg/L})(\text{analysis volume in mL})}{\text{sample volume (or mass) in mL (or g)}} = \text{mg/L}$$

The standard curve equation is of the form: $y = mx + b$

where:

- y = peak area units
- m = slope of the standard curve [X Coefficients(s)]
- x = mg of TMF/L
- b = y-intercept (Constant)

Example calculation for the Low Spike LOQ 1 sample in the 20XAAP method validation:

Standard Curve: $y = 85{,}941.269006x - 20.987864$
Sample Peak Area: 1,355
Concentration from standard curve: 0.0160 mg/L

Volume for Analysis: 10 mL
Sample Volume: 8 mL

The concentration of TFM in the sample was calculated by the following equation:

$$\frac{(0.0160\ \text{mg/L})(10\ \text{mL})}{8\ \text{mL}} = 0.0200\ \text{mg/L}$$

Recovery from the Low Spike LOQ 1 sample in the 20XAAP method validation:

$$\frac{0.0200\ \text{mg/L}}{0.0200\ \text{mg/L}} \times 100 = 100\%$$

The minimum quantifiable limit (MQL) was determined from the following equation:

$$\frac{\left(\begin{array}{c}\text{lowest standard}\\ \text{concentration as mg/L)}\end{array}\right)\left(\begin{array}{c}\text{volume for}\\ \text{analysis (mL)}\end{array}\right)}{\left(\begin{array}{c}\text{volume or mass}\\ \text{sampled (mL or g)}\end{array}\right)} = \text{MQL expressed as mg/L}$$

Example for 20XAAP method validation:
Lowest standard concentration: 0.00500 mg/L
Analysis volume: 10 mL
Sample volume: 8 mL

therefore:

$$\text{MQL} = \frac{(0.00500\ \text{mg/L})(10\ \text{mL})}{(8\ \text{mL})} = 0.00625\ \text{mg/L}$$

3.2.5 Linearity

A 6-point calibration was prepared for each analysis and slope, intercept, and correlation coefficient were determined. The correlation coefficient was used to assess the linearity of the standard curves. Section 3.2.1 describes the preparation of the standard solutions.

3.2.6 Method Validations in Test Media

3.2.6.1 Freshwater

Method validations for the recovery of TFM-HP in freshwater were performed on 20 October 2014. Five samples (low spikes) of 8 mL volumes were fortified with 0.200 mL of a 2.43 mg/L solution of TFM-HP, for a nominal concentration of 0.0608 mg Total Product (T.P.)/L, and five samples (high spikes) of 8 mL volumes were fortified with 0.330 mL of a 3,030 mg/L solution of TFM-HP, for a nominal concentration of 125 mg T.P./L. Additionally, one sample (LOD spike) of 8 mL volume was fortified with 0.060 mL of a 2.43 mg/L solution of TFM-HP, for a nominal concentration of 0.0182 mg T.P./L. The remaining two samples consisted of matrix only (i.e., 8 mL of control freshwater). The samples

were diluted with methanol and further diluted, if necessary, with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to vials for analysis by HPLC-UV.

3.2.6.2 20XAAP

Method validations for the recovery of TFM in 20XAAP were performed on 27 August 2014. Five samples (low spikes) of 8 mL volumes were fortified with 0.200 mL of a 0.800 mg/L solution of TFM, for a nominal concentration of 0.0200 mg a.i./L. Five samples (high spikes) of 8 ml volumes were fortified with 0.200 mL of a 5,010 mg/L solution of TFM, for a nominal concentration of 125 mg a.i./L. Additionally, one sample (LOD spike) of 8 mL volume was fortified with 0.060 mL of a 0.800 mg/L solution of TFM, for a nominal concentration of 0.00600 mg a.i./L. The remaining two samples consisted of matrix only (i.e., 8 mL of control 20XAAP). The samples were diluted with methanol and further diluted, if necessary, with 20:80 methanol:water to a concentration that was within the range of the standard curve (0.00500 to 0.200 mg/L). The samples were then transferred to vials for analysis by HPLC-UV.

3.2.7 Storage Stability

Three replicates of the TFM-HP low spike level (0.0608 mg T.P./L) and three replicates of the TFM-HP high spike level (125 mg T.P./L) from the freshwater method validation were analyzed after seven days of refrigerator storage. Three replicates of the TFM low spike level (0.0200 mg a.i./L) and three replicates of the TFM high spike level (125 mg a.i./L) from the 20XAAP method validation were analyzed after seven days of refrigerator storage.

Stability of TFM and TFM-HP test substance stock solutions was verified on 22 October 2014 by analysis of duplicate samples of selected stock solutions, spiking solutions, and a solution used for certificate of analysis determination.

3.2.8 Statistics

Calculations (e.g., percent area, percent difference, mean, and standard deviation) were performed using Microsoft Excel 2010, and intermediate values were not rounded during the calculations. Since Microsoft Excel was run in full precision mode, values represented in the raw data and report may be slightly different when calculated by hand.

5.0 CONCLUSIONS

This method is applicable to the assay of TFM and TFM-HP in the two ecotoxicology media (freshwater and 20XAAP) tested. The accuracy, precision, recovery, and linearity data have shown this method to be acceptable for TFM and TFM-HP. Prepared sample solution stability after at least seven days refrigerator storage was acceptable for TFM and TFM-HP.

This report satisfies the data requirement for EU SANCO\3029\99 rev.4.

6.0 DEVIATIONS

None.

7.0 REFERENCES

(1) European Commission Working Document SANCO/3029/99 rev 4. Residues: guidance for generating and reporting methods of analysis in support of pre-registration data requirements for annex II (part A, section 4) and annex III (part A, section 5) of directive 91/414.

(2) American Society for Testing and Materials (ASTM). 1998. Standard Guide for Conducting Static Toxicity Tests with Lemna gibba G3. ASTM Designation E1415-91 (Reapproved 1998).