Study Title

NF-180 - Validation of the Analytical Method for the Determination of a Test Substance in Sediment

Data Requirements

OCSPP Guideline 850.6100 (2012)
SANCO/3029/99 rev 4 (2000)

1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of NF-180 in artificial and marine sediment. The method was validated (8 to 18 August 2017) to quantify the concentrations of NF-180 present in recovery samples prepared in marine sediment and artificial sediment. 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 in artificial sediment and marine sediment by fortification with NF-180 at concentrations of 0.0500 (LOQ), 0.500 (10X LOQ), and 100 (High) mg/kg. Fortified recovery samples were extracted with 90/10/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) and diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v) followed by a Matrix Blank diluent, as appropriate. All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS).

The study was initiated on 20 July 2017, 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 8 to 18 August 2017 at Smithers Viscient (SMV), located in Wareham, Massachusetts. All original raw data, the protocol, and the final report produced during this study are stored in Smithers Viscient's archives 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 "Validation of the Analytical Method for the Determination of a Test Substance in Sediment" (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 850.6100 (U.S. EPA, 2012).

2.2 Test Substance

The test substance, NF-180, was received on 23 June 2015 from Nippon Soda Co., Ltd., Tokyo, Japan. The following information was provided:

Name:	NF-180
Lot No.:	TRED-001
CAS No.:	1314008-27-9
Purity:	99.1% (Certificate of Analysis, Appendix 2)
Expiration Date:	19 July 2018

Upon receipt at Smithers Viscient, the test substance (SMV No. 7725) was stored in a freezer in a 1-L Nalgene bottle. Concentrations were adjusted for the purity of the test substance.

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

2.3 Reagents

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

2.4 Instrumentation and Laboratory Equipment

1.	Instrument:	AB MDS Sciex API 4000 mass spectrometer equipped with an AB MDS Sciex ESI Turbo V source Shimadzu LC-20AD binary pumps Shimadzu DGU-20A3 vacuum degasser Shimadzu DGU-20A5R vacuum degasser Shimadzu SIL-20ACHT autosampler Shimadzu CTO-20AC column oven Shimadzu CBM-20A communications bus Analyst version 1.4.2 software for data acquisition
2.	Balance:	Mettler PJ-3000
3.	Shaker Table:	VWR 3500STD Analog Shaker Table
4.	Centrifuge:	Beckman Allegra X-12 Centrifuge
5.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, Teflon centrifuge tubes, 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 Matrices

The matrices used during this method validation were artificial sediment and marine sediment.

Artificial Sediment

Artificial sediment was the substrate used in the exposure. The artificial sediment (Smithers Viscient Batch No. 032217 was prepared according to OECD Guideline No. 218 (OECD, 2004) by mixing the following components (based on dry weight): 2.8 kg sphagnum peat, 11.2 kg kaolin clay, and 42 kg fine sand (i.e., 5.0, 20, and 75%, respectively).

Sediment During Testing		
Smithers Viscient Batch No.:	032217	
Sediment component processing:	Prior to being used in the sediment preparation, the peat was conditioned for seven days in laboratory well water. Over this period, 160 grams of powdered $CaCO_3$ was mixed into the peat suspension in order to increase the pH. The pH of the peat suspension increased from 3.2 to 5.7 during the conditioning period. The peat was then removed from suspension using a fine-mesh net. All of the sediment components were then blended together in a large-scale laboratory mixer. A total of 11.2 liters of laboratory well water was also added to the sediment components during the mixing process.	
Sediment Characterization by Agvise Laboratories, Northwood, North Dakota:	Percent organic carbon:	2.0%
	Particle size distribution:	82% sand 4% silt 14% clay
	Percent water holding capacity (at 1/3 Bar):	14.9%
Sediment Characterization at SMV:	Percent solids:	80.49%
	pH	7.2

Representative samples of the sediment were analyzed periodically for the presence of pesticides, PCBs, and toxic metals by Eurofins Lancaster Laboratories Environmental, Lancaster, Pennsylvania. None of these compounds have been detected at concentrations that are considered toxic in any of the samples analyzed, in agreement with ASTM (2007) standard practice (Appendix 3).

Marine Sediment

The natural, marine sediment used during this study was collected from Sequim Bay, Sequim, Washington. Prior to characterization and use in testing, the sediment was wet pressed through a 0.25-mm sieve to remove large particles and indigenous organisms. A sample of the sieved sediment was characterized by Agvise Laboratories, Northwood, North Dakota, as having a mean (n = 3) percent organic carbon of 3.7%, a particle size distribution of 29% sand, 36% silt, and 35% clay and a pH of 7.6 and a percent moisture at 1/3 bar (water holding capacity) of 72.5%. A percent solids of 30.12% was also determined by Smithers Viscient.

Representative samples of the sediment were analyzed periodically for the presence of pesticides, PCBs, and toxic metals by GeoLabs, Inc., Braintree, Massachusetts. None of these compounds have been detected at concentrations that are considered toxic in any of the samples analyzed, in agreement with ASTM (2007) standard practice (Appendix 3).

2.6 Preparation of Liquid Reagent and Mobile Phase Solutions

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 20/80 acetonitrile/purified reagent water (v/v) liquid reagent solution was prepared by combining 200 mL of acetonitrile and 800 mL of purified reagent water. The solution was mixed well using a stir bar and stir plate for five minutes.

A 90/10/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) liquid reagent solution was typically prepared by combining 1125 mL of acetonitrile, 125 mL of purified reagent water, and 1.25 mL of formic acid. The solution was mixed using a stir bar and stir plate for five minutes.

A 30/30/40 acetonitrile/methanol/purified reagent water (v/v/v) autosampler needle wash solution was 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 each separate validation. For future testing, the actual volumes and masses used may be scaled up or down as necessary.

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

Primary Stock ID	Amount of Substance Weighed (g), Net Weight	Amount of Substance Weighed (g), as Active Ingredient	Stock Solvent	Final Volume (mL)	Primary Stock Concentration (mg/L)	Primary Stock Use
7725EB	0.2535	0.2512	Acetonitrile	25.0	10,000	Secondary stock solutions
7725EA	0.0506	0.0501	Acetonitrile	50.0	1,000	Secondary stock solution

Secondary stock solutions were 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
7725EB	10,000	0.0500	50.0	Acetonitrile	7725EB-1	10.0	LOQ- and 10X LOQ-level recovery samples
		5.00			7725EB-3	1,000	High-level recovery samples
7725EA	1000	0.500	50.0	Acetonitrile	7725EA-2	10.0	Sub-stock solution

A sub-stock solution was 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 (µg/L)	Stock Use
7725EA-2	10.0	0.0500	50.0	Acetonitrile	Ana Stk 1	10.0	Calibration standards

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

The effects of matrix enhancement or suppression were evaluated through the assessment of matrix-matched and solvent-based calibration standards in the following manner. Two sets of calibration standards were prepared. One set was prepared in 20/80 acetonitrile/purified reagent water (v/v) and a second set was prepared in a matrix blank diluent (see Section 2.10). Both sets of calibration standards were prepared in the same manner by fortifying with the 10.0 µg/L sub-stock solution to yield concentrations of 0.0100, 0.0200, 0.0400, 0.0600, 0.0800, and 0.100 µg/L.

2.9 Sample Fortification and Preparation

The recovery samples were prepared in two different matrices (artificial sediment and marine sediment) with NF-180 at concentrations of 0.0500 (LOQ), 0.500 (10X LOQ), and 100 (High) mg/kg. Recovery samples for both matrices were prepared separately ("de novo") at these concentrations. Five replicates were produced for each concentration level. Five samples were left unfortified to serve as controls and were extracted in the same fashion as the LOQ concentration recovery samples. One additional sample was prepared to serve as a matrix blank, for the preparation of matrix-matched standards and for the dilution of High-concentration recovery samples into matrix-matched diluent. In addition, two reagent blanks were prepared and processed in the same manner as the control samples. While the control samples and reagent blanks were not fortified with test substance, they were fortified with the same quantity of acetonitrile as the LOQ samples. The preparation procedure for each separate matrix is outlined in the tables below.

Artificial sediment recovery samples

Sample ID 12681-6134-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Dry Weight (g)	Fortified Concentration (µg/kg)
47	Matrix Blank	NA[a]	0.0250[b]	5.00	0.00
48 & 49	Reagent Blank	NA	0.0250[b]	0.00	0.00
50, 51, 52, 53, & 54	Control	NA	0.0250[b]	5.00	0.00
55, 56, 57, 58, & 59	LOQ	10.0	0.0250	5.00	50.0
60, 61, 62, 63, & 64	10X LOQ	10.0	0.250	5.00	500
65, 66, 67, 68, & 69	High	1000	0.500	5.00	100,000

[a] NA = Not Applicable
[b] Reagent blanks and control samples were dosed with acetonitrile.

Marine sediment recovery samples:

Sample ID 12681-6134-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/kg)
70	Matrix Blank	NA[a]	0.0250[b]	5.00	0.00
71 & 72	Reagent Blank	NA	0.0250[b]	5.00	0.00
73, 74, 75, 76, & 77	Control	NA	0.0250[b]	5.00	0.00
78, 79, 80, 81, & 82	LOQ	10.0	0.0250	5.00	50.0
83, 84, 85, 86, & 87	10X LOQ	10.0	0.0250	5.00	500
88, 89, 90, 91, & 92	High	1000	0.500	5.00	100,000

[a] NA = Not Applicable
[b] Reagent blanks and control samples were dosed with acetonitrile.

2.10 Dilution of Samples

Sediment Extraction:

A 20-mL aliquot of 90/10/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) was added to each control, reagent blank, matrix blank, and recovery sample (5.00 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 50-mL volumetric flasks. The extraction and centrifugation procedures were repeated with an additional 20-mL aliquot of 90/10/0.1 acetonitrile/purified reagent water/formic acid (v/v/v). The extracts were combined, taken to volume (50 mL) with 90/10/0.1 acetonitrile/purified reagent water/formic acid (v/v/v) and mixed well. The recovery sample extracts were further diluted into the calibration standard range with 20/80 acetonitrile/purified reagent water (v/v). The 10X LOQ and High-level recovery samples

were further diluted with the matrix blank diluent. The extraction and dilution procedures are detailed below.

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC parameters:

Column:	Supelco Ascentis Express C18, 2.7 µm, 2.1 × 100 mm
Mobile Phase A:	0.1% formic acid in reagent grade water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.010	0.400	95.0	5.00
0.50	0.400	90.0	10.0
6.00	0.400	0.00	100
7.00	0.400	0.00	100
7.10	0.400	95.0	5.00
8.50	0.400	95.0	5.00

Run Time:	8.5 minutes
Autosampler Wash Solvent:	30/30/40 acetonitrile/methanol/purified reagent water (v/v/v)
Column Temperature:	40 °C
Sample Temperature:	5 °C
Injection Volume:	50 µL
Retention Time:	approximately 5.2 minutes

MS parameters:

Instrument:	MDS Sciex API 5000 mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5000 V
Scan Type:	MRM
Dwell Time:	200 milliseconds
Source Temperature:	500 °C
Curtain Gas:	20.0
Ion Source – Gas 1 / Gas 2:	35.0 / 63.0
Collision Gas:	12.0
Collision Cell Entrance Potential:	12.0
Declustering Potential:	78.0
Resolution Q1/Q3:	Unit/Unit

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses (amu):	348.1/330.0	348.1/180.2
Collision Energy:	35.0	41.0
Collision Cell Exit Potential:	7.00	13.0

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.

2.11.2 Preparation of Calibration Standard Curve

Two sets of calibration standards for both matrix-matched and solvent-based standards (for four sets in total) were analyzed with each recovery sample set; one set prior of each to analysis of the recovery samples, and the second set of each immediately following the analysis of the recovery samples. Injection of samples and calibration standards onto the LC-MS/MS system was performed by programmed automated injection.

2.12 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) and relative standard deviation (RSD or coefficient of variation (CV)) calculated for the observed recovery values. The RSD for the recovery samples should be 10% or less. Specificity of the method was determined by examination of the control samples for peaks at the same retention times as NF-180 that 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.

2.13 Limit of Quantitation (LOQ)

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

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

The Limit of Detection (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 Method Detection Limit (MDL) was defined as the lowest concentration in test samples that can be detected based on the concentration of the low 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/kg) 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.

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

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

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

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 mass, mL/g)
A	=	analytical result (µg/kg), 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 low 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 (0.0100 µg/L)
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, 2500)
MDL	=	method detection limit reported for the analysis (0.0100 µg/L × 2500 = 25.0 µg/kg)

The LOD was calculated using the following equation:

$$(4)\quad LOD = (3\times(N_{ctl}))/Resp_{LS}) \times Conc_{LS} \times DF_{CNTL}$$

where:

N_{ctl}	=	mean noise in height of the control samples (or blanks)
$Resp_{LS}$	=	mean response in height of the two low calibration standards
$Conc_{LS}$	=	concentration of the low calibration standard
DF_{CNTL}	=	dilution factor of the control samples (smallest dilution factor used, i.e., 2500)
LOD	=	limit of detection for the analysis

PROTOCOL DEVIATIONS

No deviations from the protocol occurred during this study.