Study Title

TZ-5 - Validation of the Analytical Method for the Determination of a Test Substance in Aqueous Solutions

Data Requirements

OCSPP Guideline 850.6100
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Laboratory Project ID

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1.0 INTRODUCTION

The purpose of this study was to validate an analytical method used to determine the content of TZ-5 in aqueous solutions. The method was validated (4 to 16 November 2016) to quantify the concentrations of TZ-5 present in recovery samples prepared in laboratory well water, fortified laboratory well water, and Algal Assay Procedure (AAP) medium. 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 by fortification of laboratory well water, fortified laboratory well water, and Algal Assay Procedure (AAP) medium with TZ-5 at concentrations of 0.100 (limit of quantitation, LOQ) and 100,000 (high) µg/L. Recovery samples were diluted with 20:80 acetonitrile:purified reagent water (v:v) for a final composition of 18:10:72 acetonitrile: test matrix:purified reagent water (v:v:v). The high-level recovery samples were further diluted into the calibration range with 18:10:72 acetonitrile:test matrix:purified reagent water (v:v:v). All samples were analyzed using liquid chromatography with tandem mass spectrometry detection (LC/MS/MS).

The study was initiated on 14 October 2016, 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 4 to 16 November 2016 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 "TZ-5 - Validation of the Analytical Method for the Determination of a Test Substance in Aqueous Solutions" (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, TZ-5, was received on 28 January 2016 from Nisso America Inc., New York, New York. The following information was provided:

Name:	TZ-5
Lot No.:	31-15207-T.SUGIURA
CAS No.:	33452-21-0
Purity:	99.4% (Certificate of Analysis, Appendix 2)
Recertification Date:	22 June 2017(1/2 previous stability interval)

Upon receipt at Smithers Viscient, the test substance (SMV No. 8075) was stored in a freezer in the original container. Concentrations were adjusted for the purity of the test substance.

Verification of the test and reference 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 water:	Fisher Chemical, reagent grade
2.	0.1% Formic acid in acetonitrile:	Fisher Chemical, reagent grade
3.	Methanol:	EMD, reagent grade
4.	Acetonitrile:	EMD, reagent grade
5.	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:	MDS Sciex API 5000 mass spectrometer equipped with an ESI Turbo V ion source Shimadzu 20AD/SIL-20ACHT autoinjector Shimadzu 20AD/DGU-20A3V vacuum degasser Shimadzu 20AD/DGU-20A5R vacuum degasser Shimadzu LC-20AD solvent delivery pumps Shimadzu 20AD/CTO-20A column compartment Shimadzu CBM-20A/228-40512-32 Communications bus Analyst 1.4.2 software for data acquisition
2.	Balance:	Mettler Toledo XSE205DU
3.	Laboratory equipment:	Positive displacement pipets, volumetric flasks, disposable glass vials, disposable glass pipets, 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 laboratory well water, fortified laboratory well water, and Algal Assay Procedure (AAP) medium.

Laboratory well water information:

Laboratory well water consists of a mixture of unadulterated water from a 100-meter bedrock well and de-chlorinated Town of Wareham, Massachusetts well water. This mixture was pumped into a 5700-liter polyethylene tank that was continuously circulated through de-gassing chambers where it was aerated to ensure proper equilibration of dissolved gases with the laboratory atmosphere.

Fortified laboratory well water information:

Fortified laboratory well water was prepared in 3410-L batches by fortifying laboratory well water with a mixture of salts (U.S. EPA, 2002). The well water was a mixture of unadulterated water from 100-meter bedrock well and dechlorinated Town of Wareham well water, which is considered soft with a typical hardness of < 160 mg (as $CaCO_3$). The components used to formulate the medium are listed in the table below.

Water Type	Salts required (mg/L)			
	$NaHCO_3$	$CaSO_4$ H_2O	$MgSO_4$	KCl
Hard	119	82	82	3.8

Typical water quality parameter ranges for fortified laboratory well water are listed in the table below.

pH	Alkalinity (mg/L $CaCO_3$)	Hardness (mg/L $CaCO_3$)	Conductivity µS/cm
7.9 to 8.3	80 to 130	160 to 190	600 to 900

Algal Assay Procedure (AAP) information:

Algal Assay Procedure (AAP) medium was prepared with sterile, deionized water. The components used to formulate the medium are listed in the table below. If necessary, the pH of the medium was adjusted to pH 7.5 ± 0.1 with dilute hydrochloric acid or sodium hydroxide.

Compound	Concentration
$NaNO_3$	25.5 mg/L
$MgCl_2 \cdot 6H_2O$	12.16 mg/L
$CaCl_2 \cdot 2H_2O$	4.41 mg/L
$MgSO_4 \cdot 7H_2O$	14.7 mg/L
$K_2HPO_4 \cdot 3H_2O$	1.368 mg/L
$NaHCO_3$	15.0 mg/L
H_3BO_3	185.5 µg/L
Na_2SeO_4	1.88 µg/L
$MnCl_2 \cdot 4H_2O$	415.4 µg/L
$ZnCl_2$	3.270 µg/L
$CoCl_2 \cdot 6H_2O$	1.43 µg/L
$CuCl_2 \cdot 2H_2O$	0.012 µg/L
$Na_2MoO_4 \cdot 2H_2O$	7.26 µg/L
$FeCl_3 \cdot 6H_2O$	160 µg/L
$Na_2EDTA \cdot 2H_2O$	300 µg/L
$Na_2SiO_3 \cdot 9H_2O$	202.4 mg/L

2.6 Preparation of Liquid Reagent 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 typically 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 18:10:72 acetonitrile:test matrix:purified reagent water (v:v:v) liquid reagent solution was typically prepared by combining 180 mL of acetonitrile, 100 mL of test matrix, and 720 mL of purified reagent water. 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 rinse solvent 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 the 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
8075K	0.0504	0.0501	Acetonitrile	50.0	1000	Secondary stock solution and high concentration recovery samples
8075J	0.0504	0.0501		50.0	1000	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
8075K	1000	0.500	50.0	Acetonitrile	8075K-1	10.0	Sub-stock solution
8075J	1000	0.500	50.0		8075J-1	10.0	Sub-stock solution

Sub-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 (µg/L)	Stock Use
8075K-1	10.0	0.0500	50.0	Acetonitrile	Tech Stk 1	10.0	LOQ recovery samples
8075J-1	10.0	0.0500	50.0		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 18:10:72 acetonitrile:test matrix:purified reagent water (v:v:v). 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 0.00500, 0.0100, 0.0250, 0.0350, 0.0500, and 0.100 µg/L.

2.9 Sample Fortification and Preparation

The recovery samples were prepared in three different matrices (laboratory well water, fortified laboratory well water and AAP medium) with TZ-5 at concentrations of 0.100 (LOQ) and 100,000 (High) µg/L. Recovery samples for all three 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 diluted in the same fashion as the LOQ concentration recovery samples. In addition, two reagent blanks were prepared and processed in the same manner as the control samples. The preparation procedure for each separate matrix is outlined in the tables below.

Laboratory well water recovery samples

Sample ID 12681-6170-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
52 & 53	Reagent Blank	NA[a]	NA	5.00[b]	0.00
54, 55, 56, 57 & 58	Control	NA	NA	5.00	0.00
59, 60, 61, 62 & 63	LOQ	0.0100	0.0500	5.00	0.100
64, 65, 66, 67 & 68	High	1000	0.500	5.00	100,000

[a] NA = Not Applicable.
[b] Dilution solvent: 20:80 acetonitrile:purified reagent water (v:v).

Fortified laboratory well water recovery samples

Sample ID 12681-6170-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
86 & 87	Reagent Blank	NA[a]	NA	5.00[b]	0.00
88, 89, 90, 91 & 92	Control	NA	NA	5.00	0.00
93, 94, 95, 96 & 97	LOQ	0.0100	0.0500	5.00	0.100
98, 99, 100, 101 & 102	High	1000	0.500	5.00	100,000

[a] NA = Not Applicable.
[b] Dilution solvent: 20:80 acetonitrile:purified reagent water (v:v).

Algal Assay Procedure (AAP) recovery samples

Sample ID 12681-6170-	Sample Type	Stock Concentration (mg/L)	Fortification Volume (mL)	Final Volume (mL)	Fortified Concentration (µg/L)
69 & 70	Reagent Blank	NA[a]	NA	5.00[b]	0.00
71, 72, 73, 74 & 75	Control	NA	NA	5.00	0.00
76, 77, 78, 79 & 80	LOQ	0.0100	0.0500	5.00	0.100
81, 82, 83, 84 & 85	High	1000	0.500	5.00	100,000

[a] NA = Not Applicable.
[b] Dilution solvent: 20:80 acetonitrile:purified reagent water (v:v).

2.10 Dilution of Samples

To minimize the potential for losses of the test substance during processing, the aqueous test samples were not sub-sampled prior to dilution. The first dilution with 20:80 acetonitrile:purified reagent water (v:v) was performed by the addition of the reagent to the entire volume of the aqueous sample in the container in which it was fortified to a final composition of 18:10:72 acetonitrile:test matrix:purified reagent water (v:v:v). The high concentration recovery samples were subsequently diluted into the calibration standard range with 18:10:72 acetonitrile:test matrix:purified reagent water (v:v:v) prior to analysis. The dilution procedures are outlined in the tables below.

Laboratory Well Water

Sample ID 12681-6170-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
52 & 53	Reagent Blank	0.00	5.00	50.0	NA[c]	NA	NA	NA	10.0
54, 55, 56, 57 & 58	Control	0.00	5.00	50.0	NA	NA	NA	NA	10.0
59, 60, 61, 62 & 63	LOQ	0.100	5.00	50.0	NA	NA	NA	NA	10.0
64, 65, 66, 67 & 68	High	100,000	5.00	50.0	0.0500	50.0	0.0400	10.0	2,500,000

[a] Diluted with 20:80 acetonitrile:purified reagent water (v:v).
[b] Diluted with 18:10:72 acetonitrile:laboratory well water:purified reagent water (v:v:v).
[c] NA = Not Applicable.

Fortified laboratory well water

Sample ID 12681-6170-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
86 & 87	Reagent Blank	0.00	5.00	50.0	NA[c]	NA	NA	NA	10.0
88, 89, 90, 91 & 92	Control	0.00	5.00	50.0	NA	NA	NA	NA	10.0
93, 94, 95, 96 & 97	LOQ	0.100	5.00	50.0	NA	NA	NA	NA	10.0
98, 99, 100, 101 & 102	High	100,00	5.00	50.0	0.0500	50.0	0.0400	10.0	2,500,000

[a] Diluted with 20:80 acetonitrile:purified reagent water (v:v).
[b] Diluted with 18:10:72 acetonitrile:fortified laboratory well water:purified reagent water (v:v:v).
[c] NA = Not Applicable.

Algal Assay Procedure (AAP) medium recovery samples

Sample ID 12681-6170-	Sample Type	Fortified Concentration (µg/L)	Sample Volume (mL)	Final Volume[a] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Sample Volume (mL)	Final Volume[b] (mL)	Dilution Factor
69 & 70	Reagent Blank	0.00	5.00	50.0	NA[c]	NA	NA	NA	10.0
71, 72, 73, 74 & 75	Control	0.00	5.00	50.0	NA	NA	NA	NA	10.0
76, 77, 78, 79 & 80	LOQ	0.100	5.00	50.0	NA	NA	NA	NA	10.0
81, 82, 83, 84 & 85	High	100,000	5.00	50.0	0.0500	50.0	0.0400	10.0	2,500,000

[a] Diluted with 20:80 acetonitrile:purified reagent water (v:v).
[b] Diluted with 18:10:72 acetonitrile: Algal Assay Procedure (AAP) medium:purified reagent water (v:v:v).
[c] NA = Not Applicable.

2.11 Analysis

2.11.1 Instrumental Conditions

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

LC parameters:

Column:	Waters XBridge C18, 2.5 µm, 2.1 × 50 mm
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile

Gradient:

Time (min.)	Flow rate (mL/min.)	Solvent A (%)	Solvent B (%)
0.10	0.250	95.0	5.00
1.00	0.250	95.0	5.00
3.50	0.250	0.00	100
4.50	0.250	0.00	100
4.60	0.250	95.0	5.00
6.50	0.250	95.0	5.00

Run Time:	6.5 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:	100 µL
Retention Time:	approximately 3.9 minutes

MS parameters:

Instrument:	MDS Sciex API 5000 mass spectrometer
Ionization Mode:	Positive (+) ESI
Ion Spray Voltage:	5500 V
Scan Type:	MRM
Dwell Time:	500 milliseconds
Source Temperature:	500 °C
Curtain Gas:	20.00
Ion Source – Gas 1 / Gas 2:	50.00 / 50.00
Collision Gas:	10.00
Collision Cell Entrance Potential:	10.00
Declustering Potential:	40.00
Resolution Q1/Q3:	Low/Low

	Primary Transition	Confirmatory Transition
Q1/Q3 Masses:	191.20/145.10 amu	191.20/117.10 amu
Collision Energy:	16.00	25.00
Collision Cell Exit Potential:	10.00	10.00

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 non-matrix-matched standards (for four sets in total) were analyzed with each recovery 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 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) were considered acceptable.

The precision was reported in terms of the relative standard deviation (RSD) for the recovery samples and retention times. RSD values less than 20% were considered acceptable for the recovery samples (with less than 10% considered ideal). Specificity of the method was determined by examination of the control samples for peaks at the same retention times as TZ-5 which 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. The lowest concentration on the calibration curve for this testing is approaching the quantitation limits of the instrumentation, primarily the LC/MS/MS detector. Linearity at low detector sensitivities often requires a reduced calibration curve range. Generally speaking, larger calibration curve ranges

have a positive impact on the results of testing due to the fact that reduction of the variables associated with any analysis particularly those related to handling and stability will typically result in a more robust set of data. For the reasons stated above, the calibration curve for this testing is generated using a polynomial (quadratic) fit.

2.13 Limit of Quantitation

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 LOQ.

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

The Limit of Detection (LOD) was calculated using three times the signal-to-noise value of the control samples. Representative calculations for the LOD can be found in Calculations.

The Method Detection Limit (MDL) was defined as the lowest concentration in test samples which 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 Calculations.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg/L) in the calibration standards against the peak area ratio of the calibration standards. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of the test substance within each recovery sample was determined using the regression coefficients from the quadratic equation, the peak area ratio of the recovery sample, and the dilution factor. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

$$(1) \qquad y = ax^2 + bx + c$$

$$(2) \qquad DC(x) = \frac{-b + \sqrt{b^2 - 4aC}}{2a}$$

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

where:

y	=	detector response (peak area) for analyte
a, b and c	=	regression constants
DC (x)	=	detected concentration (µg/L) in the sample
C	=	constant c minus the peak area; C = (c - y)
DF	=	dilution factor (the final sample volume divided by the original sample volume)
A	=	concentration of the analyte in the original sample

The LOD was calculated using the following equation:

$$LOD = (3x(SN_{ctl}))/Resp_{LS}) \times Conc_{LS}$$

where:

SN_{ctl}	=	Mean signal to noise in height of the control samples (or blanks)
$Resp_{LS}$	=	Mean Response in height of the two low calibration standards (0.00500 µg/L)
$Conc_{LS}$	=	Concentration of the low calibration standard
LOD	=	limit of detection for the analysis

The MDL was calculated using the following equation:

$$(4) \qquad MDL = MDL_{LCAL} \times DF_{CNTL}$$

where:

MDL_{LCAL} = The lowest concentration calibration standard (i.e., 0.00500 µg/L)
DF_{CNTL} = Dilution factor of the control samples (smallest dilution factor used, i.e., 10.0)
MDL = Method detection limit reported (0.00500 µg/L × 10.0 = 0.0500 µg/L)