TITLE

Validation of Residue Analytical Method for Determination of NF-171 and its Metabolites TZ-1E and TZ-2 in Surface Water

TEST GUIDELINE

OCSPP 850.6100

AUTHOR(S)

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STUDY COMPLETION DATE

August 29, 2017

PERFORMING LABORATORY

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SUMMARY

Objective
The scope of this study was, in compliance with the principles of Good Laboratory Practices (GLP), to validate the method for analysis of NF-171, TZ-1E and TZ-2 in surface water according to the guidance document SANCO/825/00 rev. 8.1 and OCSPP850.6100, with a limit of quantification (LOQ) of 0.05 µg/L for NF-171 and TZ-1E and 0.1 µg/L for TZ-2.

Analysis	**LOQ***	**Method**
NF-171 and TZ-1E	0.05 µg/L	LC-MS/MS
TZ-2	0.1 µg/L	

LC-MS/MS: High Performance Liquid Chromatography coupled with tandem Mass Spectrometry detection.
*Expressed as individual analytes

Method Summary
Residues of NF-171, TZ-1E and TZ-2 in water were diluted in acetonitrile prior to quantification by LC-MS/MS.

The limit of quantification will be 0.05 µg/L for NF-171 and TZ-1E and 0.1 µg/L TZ-2. The limit of detection for each analyte will be at least 30 % of the LOQ.

Selectivity
NF-171, TZ-1E and TZ-2 were analysed using LC-MS/MS considered as a highly selective method monitoring two specific transitions.

Specificity

Two (2) control samples were analysed to investigate residue level and to check for any background interferences at the expected retention time. In addition at least one (1) reagent blank (sample extraction procedure without matrix) was run within the course of the study.

For both mass transitions, control samples showed no significant interferences (above 30 % of LOQ) at the retention time of NF-171, TZ-1E and TZ-2 in surface water; therefore, the specificity of the method is sufficiently proven.

Matrix Effect
Due to non-significant matrix effect (< ±20%) on signal, quantification using standard solutions prepared in solvent was performed for all analytes.

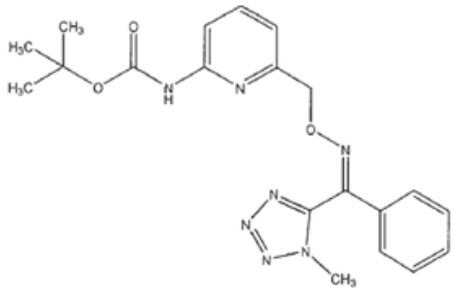
Linearity
The linearity of the response was demonstrated by single determination of standards calibration prepared in solvent on at least 5 concentration levels ranging from 0.0075 ng/mL to 0.375 ng/mL for NF-171 and TZ-1E (corresponding to 0.015 µg/L to 0.75 µg/L in matrix) and from 0.015 ng/mL to 0.75 ng/mL for TZ-2 (corresponding to 0.03 µg/L to 1.5 µg/L in matrix) for analysis of surface water. This covers the range from no more than 30 % of the LOQ and above 20 % of the highest level detected in samples.

The calibration curves obtained for both mass transitions of each analytes were found linear with coefficients of correlation (R) ≥ 0.995. Linear regression was performed with 1/x weighted.

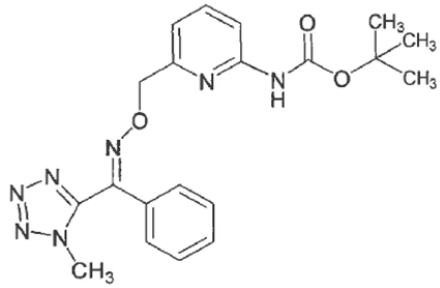
3. MATERIAL AND METHODS

3.1. Test / Reference Item

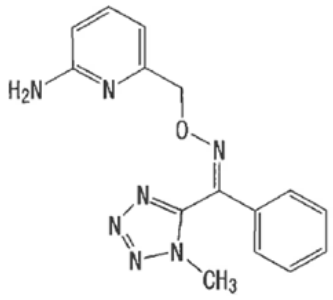
These test / reference items were used for calibration and fortification purposes.

Reference Item 1	
Common name	**NF-171 (Picarbutrazox)**
Chemical name	*tert*-butyl(6-{[(*Z*)-(1-methyl-1*H*-5-tetrazolyl)(phenyl)methylene]aminooxymethyl}-2-pyridyl)carbamate
Molecular formula	$C_{20}H_{23}N_7O_3$
Molecular weight	409.44
Structure	
Supplier	Sponsor
Batch No.	31-09142-Y.MIZOGUCHI
Purity	98.8 %
Expiration date	30 Aug 2018
Storage conditions	Between 2and 6°C*

*Certificate of analysis mentions “Keep in a refrigerator (at -1-15°C) considered as equivalent conditions

Reference Item 2	
Common name	**TZ-1E**
Chemical name	*Tert-butyl(6-{[(E)-(1-methyl-1H-5-tetrazolyl)(phenyl)methylene]aminooxymethyl}-2-pyridyl)carbamate*
Molecular formula	$C_{20}H_{23}N_7O_3$
Molecular weight	409.44
Structure	
Supplier	Sponsor
Batch No.	31-10187-T.SUGIURA
Purity	98.0 %
Expiration date	11 May 2018
Storage conditions	<-18°C*

*Certificate of analysis mentions “Keep in a freezer (at -20±10°C) considered as equivalent conditions

Reference Item 3	
Common name	**TZ-2**
Chemical name	*(Z)-O-[(6-amino-2-pyridyl)methyl](1-methyl-1H-5-tetrazolyl)(phenyl)methanone oxime*
Molecular formula	$C_{15}H_{15}N_7O$
Molecular weight	309.33
Structure	
Supplier	Sponsor
Batch No.	31-10242-T.SUGIURA
Purity	99.7 %
Expiration date	04 Mar 2018
Storage conditions	<-18°C*

*Certificate of analysis mentions “Keep in a freezer (at -20±10°C) considered as equivalent conditions

Stock solutions and working solutions of the test / reference item were stored refrigerated in capped vials protected from light. They were freshly prepared and were refrigerated after vial setting.
Usual safety precautions with chemicals were obeyed.

All specifications given on the certificate of analysis, provided by the sponsor/ supplier, are essential for correct identification of the test / reference item for use under GLP. They were not verified by the test facility and no claim of GLP compliance were made for these data, except where this is explicitly claimed on the certificate of analysis. Additional specifications for test / reference item characterisation may originate from (non-GLP) sources other than the sponsor/ supplier and are also part of the final report.

A copy of test / reference items certificates is documented in the appendix of the final report.

3.2. Test System

Table 1: Test System

Test System	Preparation	Origin	Identification
Surface Water	No specific preparation required. Specimens was thawed completely, then homogenized by hand prior taking an aliquot for analysis.	Sampled at Perrier sur Andelle in Andelle river	ECH17-0015

Quality data to demonstrate that the sample is a typical surface water in terms of its inorganic load (e.g. conductivity, hardness, pH) and its organic load (e.g. dissolved organic carbon content (DOC)) are provided in Appendix E. These data were generated out of GLP.
Specimens were stored deep frozen (approximately at -18°C) at the test facility in the dark until fortification and extraction.

3.3. Method

3.3.1. Principle

Residues of NF-171, TZ-1E and TZ-2 in water were diluted in acetonitrile prior to quantification by LC-MS/MS.

The limit of quantification will be 0.05 µg/L for NF-171 and TZ-1E and 0.1 µg/L TZ-2. The limit of detection for each analyte will be at least 30 % of the LOQ.

3.3.2. Equipment

Equivalent equipment may be used.

Table 2: Equipment

Equipment	Supplier	Model
MilliQ water system producer	Millipore	ACADEMIC A10
Scale (precision 0.1g)	Mettler-Toledo	MS304S
Scale (precision 0.01mg)	Mettler-Toledo	XS205
Automatic Pipette	Thermo	Finnpipette
Ultrasonic bath	VWR	USC300T
Syringe filter	Agilent	nylon 25 mm – 0.45 µm – Part No. 160523002
Folded filter paper	Whatman	Diameter 185 mm - Part No. 10 311 647
Laboratory glassware volumetric Class A	VWR	Various
HPLC Column	Phenomenex	Kinetex EVO C18 100x3.0 (2.6µm) Part No.00D-4725-Y0
LC Autosampler	Shimadzu	SIL-30ACMP
LC Pump	Shimadzu	LC-30AD
LC Solvent/degazing rack	Shimadzu	DGU-20A5R
LC Oven	Shimadzu	CTO-20AC
LC Reservoir Selection Valves	Shimadzu	FCV-11AL
Detector	AB Sciex	API5500 Qtrap
Software	AB SCIeX	Analyst 1.6.2

3.3.3. Reagents

Equivalent reagents may be used.

Table 3: Reagents

Reagent	Purity/ Grade	Supplier
Ultrapure Water	-	Produced by the lab.
Acetonitrile	Hipersolv Chromanorm for HPLC	VWR
Ammonium Acetate	For mass spectrmetry	Sigma Aldrich
Ammonia 25 %	EMSURE	Merck

<u>Preparation</u>
Mobile Phase “B” for HPLC analysis : 10 mM ammonium acetate + 0.1 mL 25 % ammonia + 1000 mL ultra pure water
Dissolve 770 mg of ammonium acetate in 1 L of ultrapure water into a 1 L glass bottle and add 0.1 mL of 25 % ammonia solution. Degas at least 30 min in an ultrasonic bath. Should not be stored more than 1 week at room temperature in a closed flask.

Acetonitrile / water (50/50; v/v)
Mix 500 mL of ultrapure water and 500mL of acetonitrile into a 1 L glass bottle. Should not be stored more than 1 month at room temperature in a closed flask.

3.3.4. Standard Solutions

All standard solutions (stock solutions, fortification solutions and calibration solutions) were proven to be stable for 25 days when stored a refrigerator at +2 °C to +8 °C during the validation study IF-17/04113959 (Ref. 1).

3.3.4.1. Preparation of stock solutions

Weight approximately exactly 10 mg of NF-171, TZ-1E and TZ-2 separately into a 10 mL volumetric flask and filled up to the mark with the acetonitrile. Stock solution is approximately 1 mg/mL.
Two independent stock solutions (SM1 and SM2) are prepared to perform calibration and fortification solutions independently.
Exact concentrations are calculated taking into account exact weight and purity.

3.3.4.2. Preparation of fortifications solutions

Fortification solutions were prepared as it described in the table below:

Table 4: Fortification solutions

Solution identification	Volume Taken (mL)	From solution	Dilution Solvent	Final volume (mL)	NF-171 and TZ-1E Concentration (ng/mL)	TZ-2 Concentration (ng/mL)
SF1 of SM2	0.05 0.05 0.1	SM2 NF-171 SM2 TZ-1E SM2 TZ-2	Acetonitrile	100	500	1000
SF2 of SM2	1	SF1 of SM2		10	50	100
SF3 of SM2	1	SF1 of SM2		100	5	10

3.3.4.3. Preparation of calibration solutions

Calibration solutions were prepared as it described in the table below:

Table 5: Standard solutions

Solution identification	Volume Taken (mL)	From solution	Dilution Solvent	Final Volume (mL)	NF-171 and TZ-1 E Concentration (ng/mL)	TZ-2 Concentration (ng/mL)
SF1 of SM1	0.05 0.05 0.1	SM1 NF-171 SM1 TZ-1E SM1 TZ-2	Acetonitrile/ H2O (50/50, v/v)	100	500	1000
SF2 of SM1	1	SF1 of SM1		10	50	100
SF3 of SM1	0.15	SF1 of SM1		20	3.75	7.5
SF4 of SM1	1	SF2 of SM1		20	2.5	5
SF5 of SM1	0.5	SF2 of SM1		20	1.25	2.5
SF6 of SM1	0.25	SF2 of SM1		20	0.625	1.25
SF7 of SM1	0.1	SF2 of SM1		20	0.25	0.5
SF8 of SM1	0.05	SF2 of SM1		20	0.125	0.25
SF9 of SM1	0.15	SF2 of SM1		100	0.075	0.15

Standards solutions were diluted 10-folds in acetonitrile/ultrapure water (50/50, v/v) or in control specimen final extract to prepare the standard calibration curve in solvent or in matrix at the following concentrations:

0.0075 – 0.0125 – 0.025 – 0.0625 – 0.125 – 0.25 – 0.375 ng/mL for NF-171and TZ-1E
0.015- 0.025 – 0.05 – 0.125 – 0.25 – 0.5 – 0.75 ng/mL in matrix for TZ-2

Equivalent to :

0.015 – 0.025 – 0.05 – 0.125 – 0.25 – 0.5 – 0.75 µg/L in matrix for NF-171and TZ-1E
0.03 – 0.05 – 0.1 – 0.25 – 0.5 – 1.0 – 1.5 µg/L in matrix for TZ-2

3.3.5. Analytical Procedure

3.3.5.1. Specimens fortification

Fortifications were performed on control specimens using fortification solutions as follow:

Table 6: Fortifications

NF171 and TZ-1E Fortification level (µg/L)	TZ-2 Fortification level (µg/L)	Sample Volume (mL)	Fortification solution identification	Spiking volume (mL)
0.05	0.1	5	SF3 of SM2	0.05
0.5	1.0	5	SF2 of SM2	0.05

3.3.5.2. Specimens preparation

The water specimens were filtered through a folded filter.
5 mL filtered surface water were transfered into a 10 mL volumetric flask. If required, fortification took place at this point.
The volumetric flask was filled up to the mark with acetonitrile and shaken.
Small aliquots were filtered through a syringe filter (nylon) into a sample vial and measured by LC-MS/MS.

3.3.5.3. LC-MS/MS Analysis

Method conditions

Chromatographic Conditions			
Injection:	5 µL		
Mobile Phase A: Mobile Phase B:	10 mM ammonium acetate + 0.1 mL 25 % ammonia + 1000 mL ultra pure water Acetonitrile		
Flow Rate:	0.6 mL/min		
Column	Kinetex EVO C18 100A 100x3 mm 2.6 µm		
Autosampler temperature	10°C		
Column oven temperature	25°C*		
Gradient	Time (min)	Phase A (%)	Phase B (%)
	0.0	99	1
	0.5	99	1
	2.0	55	45
	5.0	10	90
	7.0	10	90
	7.2	99	1
	9.0	99	1
Retention Times			
NF-171		Approx. 4.7 min	
TZ-1E		Approx. 4.6 min	
TZ-2		Approx. 3.4 min	
Source Parameters			
Ion Source:	Turbo Ion Spray (ESI)		
Ion Polarity	Positive		
Curtain gas	35		
Collision gas	Medium		
Ionspray voltage	5500 V		
Temperature	450°C		
Ion source gas 1	50		
Ion source gas 2	60		

Acquisition Parameters						
Analytes	Mass transition (m/z)	Declustering potential (DP) [V]	Entrance potential (EP) [V]	Collision energy (CE) [eV]	Cell exit potential (CXP) [V]	Dwell time [ms]
NF-171 and TZ-1E	410.2 → 310.2**	61	10	19	12	100
	410.2 → 107.1	61	10	33	12	100
TZ-2	310.2 → 107.1**	76	10	25	10	100
	310.2 → 80.0	76	10	55	12	100

* Oven temperature modified from 20 °C to 25°C to meet equipment specification. No impact on the study
** proposed for quantification

3.3.6. Method Management and Time Requirements

Overnight and over a weekend stopping points

The analytical procedure may be interrupted overnight and, if necessary even over a weekend after the following step:

1. Dilution

Procedural recoveries results validate storage step.

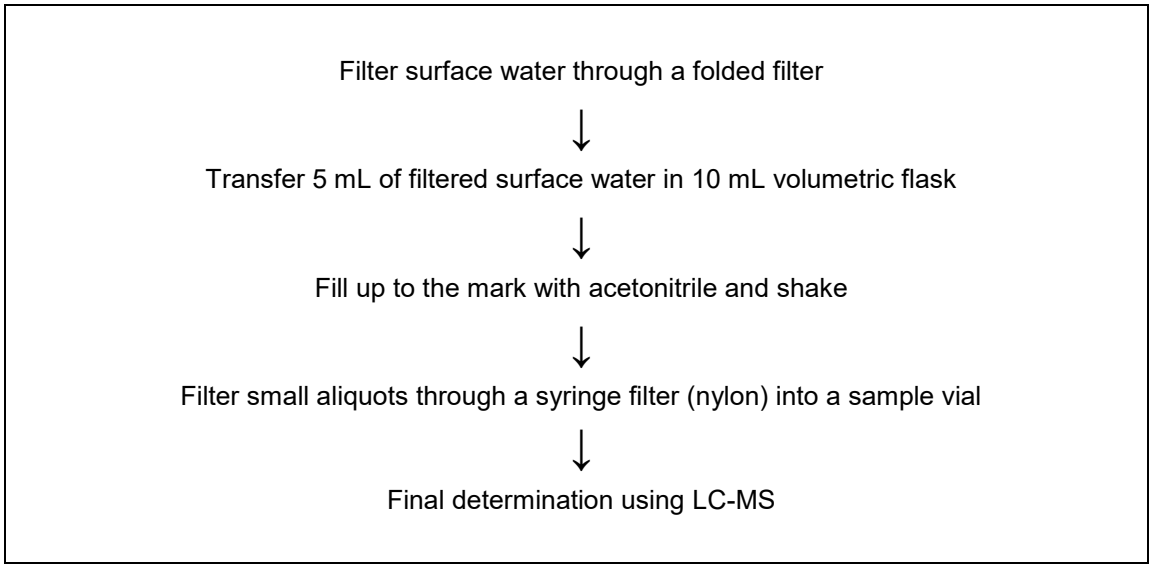
Recommended time management

1st day:	until end
Overnight	LC/MS/MS
2nd day:	calculation of results

Time Requirements

The analysis of one set of samples (= 40 samples, 1 control and 2 fortifications for recovery experiments) requires approximately 15 hours. This time includes the calculation of the results, the setup of the equipment if no special problems arise.

3.3.7. Flow chart



3.3.8. Expression of results

Amount of analyte in specimen is calculated as follow:

Calibration curve equation: y = a x + b, 1/x weighted,

- y: Analyte area
- x: Analyte conc

$$\text{Analyte amount}(\mu g/L) = c \times \frac{V_{fin}}{V} \times D$$

- c: Analyte concentration (ng/mL)
- V_{fin}: Final volume (10 mL)
- V: Sample Volume (5 mL)
- D: Dilution or concentration factor of the extract (if needed)

Recovery Calculation

$$\text{Recovery}(\%) = 100 \times \frac{\text{Analyte amount}_{\text{recovered}}}{\text{Analyte amount}_{\text{fortified}}}$$

3.3.9. Data Recording

The application programmes used to acquire and derive data for this study include Word, Excel and Analyst.

Excel sheets used for this study were verified.

4. DEVIATIONS

No deviation from the study plan and from SGS Multilab standard operating procedures (SOP) was observed.