

TITLE

Method Validation for the Determination of NF-171, TZ-1E
and TZ-2 in surface water by LC-MS/MS

TEST GUIDELINE

OCSPP 850.6100

AUTHOR(S)

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STUDY COMPLETION DATE

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PERFORMING LABORATORY

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LABORATORY PROJECT ID

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Abstract

The aim of the study was to validate an analytical method for the determination of NF-171, TZ-1E and TZ-2 in surface water. Additionally the storage stability of NF-171, TZ-1E and TZ-2 in surface water was examined for a duration of 28 days. After 0, 7, 14 and 28 days of storage at ≤ -18 °C, one set of specimens was analysed consisting of 3* control specimens (2 control specimens were freshly fortified at LOQ and 10x LOQ with the respective analyte) as well as 2 stored specimens, fortified with the respective analyte at 10x LOQ at the beginning of the study.

Five specimens fortified at the limit of quantitation (LOQ) and five specimens fortified ten times higher than the LOQ were prepared for determination of NF-171, TZ-1E and TZ-2.

The LOQ was 0.05 µg/L for NF-171 and TZ-1E.

For TZ-2 the LOQ was 0.1 µg/L.

The calibration and evaluation of specimens was performed on the basis of two ion transition recordings (MRM).

	Primary Ion Transition	Secondary Ion Transition
NF-171	410 > 310	410 > 107
TZ-1E	410 > 310	410 > 107
TZ-2	310 > 107	310 > 80

2.2 Materials

SGS INSTITUT FRESENIUS GmbH, Im Maisel 14, 65232 Taunusstein, Germany will archive the following materials under GLP-conditions:

- test/reference item
 - A sample of the test/reference items will be archived until the respective expiration date or until the next GLP-authority inspection, but at least for a period of 2 years. Thereafter, the sample will be disposed in the correct way.
- Specimens
 - SGS INSTITUT FRESENIUS GmbH will store an aliquot of the specimen until the end of this study. After study completion (final report), the specimens will be disposed of in the correct way.

3 Information about the Study

3.1 Test System

Surface water from a small stream (Burbach) in Dietzhöhlztal was used as the test system.

It was given the specimen number 170474280.

3.2 Test/Reference Item

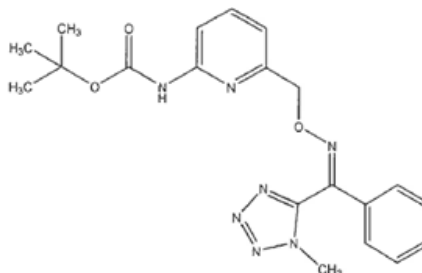
The test items, namely NF-171, TZ-1E and TZ-2 and related data were provided by the Sponsor.

The test items also acted as reference items (external quantification purposes) and were used for fortification experiments.

Picarbutrazox NF-171

Code Name:	NF-171
Lot No.:	31-09142-Y.MIZOGUCHI
Certificate No.:	160151
Chemical Name:	<i>tert</i> -butyl(6-[[<i>Z</i>]- <i>(1-methyl-1H-5-tetrazolyl)</i> (phenyl)methylene]aminooxymethyl)-2-pyridyl)carbamate

Chemical structure:

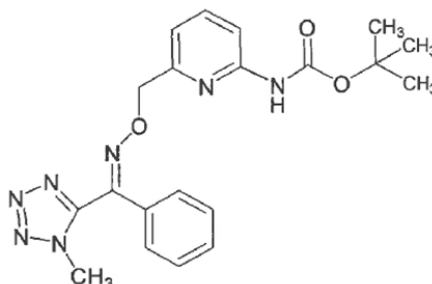


Molecular Formula:	C ₂₀ H ₂₃ N ₇ O ₃
Molecular weight:	409.44 g/mol
Appearance (at SGS-IF):	solid, white
Purity:	98.8 %
Storage Advice:	Keep in a refrigerator (2 – 8 °C at SGS-IF)
Expiry Date:	August 30, 2018
Safety Instructions:	Do not ingest, do not inhale and avoid skin contact
Caution:	Restore at ambient temperatures gradually before using

TZ-1E

Code Name: TZ-1E
 Lot. No.: 31-10187-T.SUGIURA
 Certificate No.: 160152
 Chemical Name: *Tert*-butyl(6-[[*(E)*-(1-methyl-1*H*-5-tetrazolyl)(phenyl)methylene]aminooxymethyl]-2-pyridyl)carbamate

Chemical structure:

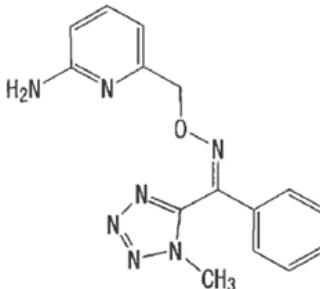


Molecular Formula: $C_{20}H_{23}N_7O_3$
 Molecular Weight: 409.44 g/mol
 Appearance (at SGS-IF): solid, white
 Purity: 98.0 %
 Storage Advice: Keep in a freezer (≤ -18 °C at SGS-IF)
 Expiry Date: May 11, 2018
 Safety Instructions: Do not ingest, do not inhale and avoid skin contact
 Caution: Restore at ambient temperatures gradually before using

TZ-2

Code Name: TZ-2
 Lot. No.: 31-10242- T.SUGIURA
 Certificate No.: 160153
 Chemical Name: (*Z*)-*O*-[(6-amino-2-pyridyl)methyl](1-methyl-1*H*-5-tetrazolyl)(phenyl)methanone oxime

Chemical structure:



Molecular Formula: $C_{15}H_{15}N_7O$
 Molecular Weight: 309.33 g/mol
 Appearance (at SGS-IF): solid, white
 Purity: 99.7 %
 Storage Advice: Keep in a freezer (≤ -18 °C at SGS-IF)
 Expiry Date: March 04, 2018
 Safety Instructions: Do not ingest, do not inhale and avoid skin contact
 Caution: Restore at ambient temperatures gradually before using

Stock solutions and working solutions of the reference items were stored between + 2 °C and + 8 °C in capped vials protected from light. Usual safety precautions with chemicals were obeyed.

4 Purpose of the Study

The aim of the study was to validate an analytical method for the determination of NF-171, TZ-1E and TZ-2 in surface water. Additionally a storage stability test for 28 days was carried out. Adsorption ought to be investigated if recoveries of the stored specimens would have been below 70 %. As all recoveries were > 70 % adsorption effects were not investigated.

The target limit of quantification (LOQ) for NF-171 and TZ-1E was 0.05 µg/L and for TZ-2 the envisaged LOQ was 0.1 µg/L.

Fortification levels of 0.05 µg/L and 0.5 µg/L were validated for NF-171 and TZ-1E; for TZ-2 the fortification levels were 0.1 µg/L and 1.0 µg/L.

The validation included analysis of two control specimens and five specimens fortified at the LOQ and five specimens fortified at 10-fold LOQ. A reagent blank as well as a blank matrix spike was also performed.

5 Analytical Phase

5.1 Principle of the Analytical Method

The surface water was filtered through a folded filter. 50 mL filtered surface water were weighed into a 100 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 HPLC-MS/MS.

5.2 Deviation from the analytical method

For determination of the storage stability specimens an aliquot of the specimens was filtered through a 0.2 µm nylon syringe filter.

5.2.1 Equipment and Materials

5.2.1.1 General Laboratory Equipment

- analytical balance	Sartorius, model CPA225D-OCE
- top loading balance	Sartorius, Model CPA 3202S
- ultra pure water unit	Merck Millipore, Model Milli-Q® 8 Direct System
- micro pipettes sizes	Gilson, model Microman, different sizes
- Single use syringe	different sizes
- pasteur pipette	glass
- volumetric flask	glass, various
- beaker	glass, various
- glass bottles	glass, various
- funnel	glass, various
- graduated cylinders	glass, various
- injection vial caps	VWR, 1.5 mL, amber with PTFE sealed
- Sample vial septum	20 mL, with steel cap and PTFE-
- folded filter	185 mm, Whatman 10311647
- syringe filter	nylon AF0-2207-52, Phenomenex

5.2.1.2 Reagents

- acetonitrile (min 99.90 %)	VWR, 83640.320
- ultra pure water	Millipore from Milli-Q® 8 Direct System
- ammonium acetate	VWR Prolabo, 84885.260
- ammonia	Merck, 1.05432

Hazard classification is based on the Globally Harmonised System (GHS):

Substance	Signal word	Hazard statements
Acetonitrile	Danger	H225 Highly flammable liquid and vapour. H302 + H312 + H332 Harmful if swallowed, in contact with skin or if inhaled. H319 Causes serious eye irritation.
Ammonium acetate	Danger	H319 Causes serious eye irritation. H335 May cause respiratory irritation H315 Causes skin irritation
ammonia	Danger	H221 Flammable gas H280 Contains gas under pressure; may explode if heated H331 Toxic if inhaled H314 Causes severe skin burns and eye damage H400 Very toxic to aquatic life

The pertinent safety instructions were observed when working with all compounds mentioned in this method.

Preparation of the Mixtures

diluent	500 mL acetonitrile + 500 mL ultra pure water
eluent A	770 mg ammonium acetate + 1000 mL ultra pure water + 0.1 mL 25% ammonia solution
eluent B	acetonitrile LCMS-grade

5.3 Final Determination by HPLC-MS/MS

The residues of NF-171, TZ-1E and TZ-2 were determined using HPLC-MS/MS equipment.

HPLC System

System:	Agilent A1290
Pump with integrated degasser:	Agilent, G 4220 A
Injection system:	CTC Analytics, PAL HTC-xt-DLW
Column oven with column selection valve:	Agilent, G 1316 C
Control module:	Agilent, G 4208 A

HPLC-Conditions

Analytical Column:	Kinetex EVO C18 100A
length:	100mm
interior diameter:	3 mm
particle size:	2.6 µm
Manufacturer:	Phenomenex
Oven temperature:	20 °C
Injection volume:	5 µL (loop)
Temp. of sample thermostat:	10 °C

Eluent A1:	10 mM ammonium acetate + 0.1 mL 25 % ammonia + 1000 mL ultra pure water
Eluent B1:	acetonitrile (LCMS grade)

Flow rate: see table below

Gradient time table:

Time [min]	Eluent A1 [%]	Eluent B1 [%]	Flow [µl/min]
0.0	99	1	600
0.5	99	1	600
2.0	55	45	600
5.0	10	90	600
7.0	10	90	600
7.2	99	1	600
9.0	99	1	600

Switching intervals: to waste: 0 – 2.5 min
to LC-MS/MS: 2.5 – 5.5 min
to waste: 5.5 – 9 min

Retention Times:

NF-171: approx. 4.6 min

TZ-1E: approx. 4.5 min

TZ-2: approx. 3.4 min

MS/MS System

System: Sciex, API 6500

Vacuum pump: Agilent, MS 40+

Data system: Sciex, Analyst, version 1.6.2

MS/MS Conditions

	NF-171 Quantifier	NF-171 Qualifier	TZ-1E Quantifier	TZ-1E Qualifier
Scan type:	MRM	MRM	MRM	MRM
Ionisation mode:	ESI +	ESI +	ESI +	ESI +
Curtain gas: [psig]	45	45	45	45
Collision gas: [psig]	10	10	10	10
Ionisation voltage: [V]	5500	5500	5500	5500
Temperature: [°C]	460	460	460	460
Declustering potential: [V]	25	25	25	25
Collision energy: [V]	19	31	19	31
Collision cell exit potential: [V]	22	12	22	12
Dwell time: [msec]	80*/50	80*/50	80*/50	80*/50
Transition used for evaluation: [m/z]	410 → 310	410 → 107	410 → 310	410 → 107

* 80 [msec] were used for the SST-Test

		TZ-2 Quantifier	TZ-2 Qualifier
Scan type:		MRM	MRM
Ionisation mode:		ESI +	ESI +
Curtain gas:	[psig]	45	45
Collision gas:	[psig]	10	10
Ionisation voltage:	[V]	5500	5500
Temperature:	[°C]	460	460
Declustering potential:	[V]	66	66
Collision energy:	[V]	25	57
Collision cell exit potential:	[V]	12	10
Dwell time:	[msec]	80	80
Transition used for evaluation:	[m/z]	310 → 107	310 → 80

5.4 Preparation of Solutions for Fortification and Calibration

The stock, fortification and calibration solutions were stored in a refrigerator at +2 °C to +8 °C immediately after preparation in capped vials protected from light. During this study, the stability of the calibration solutions (in acetonitrile / water, 50/50, v/v) and spiking solutions (in acetonitrile) was proved for 25 days.

5.4.1 Stock and Fortification Solutions

Two stock solutions of NF-171, TZ-1E and TZ-2 were prepared. From the first stock solution, the working solutions and the calibration solutions were prepared. The second stock solution was used for comparing with the first solutions at two different concentrations. These concentrations were in the range of the calibration. After successful comparison the second solution was discarded.

Example for preparation:

On 02 May 2017, stock solutions of NF-171, TZ-1E and TZ-2 were prepared in volumetric flasks. For NF-171 21.23 mg were dissolved in 20 mL of acetonitrile which resulted in a concentration of 1.048762 mg/mL, taking a purity of 98.8 % into account. For TZ-1E 20.95 mg were dissolved in 20 mL of acetonitrile which resulted in a concentration of 1.02655 mg/mL, taking a purity of 98 % into account.

For TZ-2 21.48 mg were dissolved in 20 mL of acetonitrile which resulted in a concentration of 1.070778 mg/mL, taking a purity of 99.7 % into account.

On 02 May 2017 a stock mixture (SM) containing NF-171, TZ-1E and TZ-2 was prepared by diluting 0.192 mL of NF-171, 0.195 mL of TZ-1E and 0.375 mL of TZ-2 of the stock solutions with 20 mL of acetonitrile, resulting in concentrations of 10.07 µg/mL NF-171, 10.01 µg/mL TZ-1E and 20.08 µg/mL TZ-2.

On 04 May 2017 the following fortification/spiking solutions (SP) were prepared by diluting the stock mixture (SM) with acetonitrile:

Take solution (µg/mL)	Volume (mL)	Dilute to a final volume of (mL)	Concentration NF-171 (µg/mL)	Concentration TZ-1E (µg/mL)	Concentration TZ-2 (µg/mL)	Solution name
SM	1.0	20	0.503 ^{*)}	0.500 ^{*)}	1.004 ^{*)}	SP1 ^{**)}
SP1	2.0	20	0.050 ^{*)}	0.050 ^{*)}	0.100 ^{*)}	SP2 ^{**)}

^{*)}rounded values

^{**)}used for fortification

5.4.2 Calibration Solutions

For NF-171 and TZ-1E:

On 04 May 2017, 3 mL of spiking solution SP2 (0.05 µg/mL) were dissolved in 17 mL acetonitrile/H₂O (50;50, v:v) resulting in solution ZV1 with a concentration of 7.6 ng/mL for NF-171 and 7.5 ng/mL for TZ-1E. ZV1 was used to prepare calibration solutions ranging from 0.008 ng/mL to 3.8 ng/mL.

For TZ-2:

On 04 May 2017, 3 mL of spiking solution SP2 (0.1 µg/mL) were dissolved in 17 mL acetonitrile/H₂O (50;50, v:v) resulting in solution ZV1 with a concentration of 15.1 ng/mL. ZV1 was used to prepare calibration solutions ranging from 0.015 ng/mL to 0.75 ng/mL.

Take solution (ng/mL)	Volume (mL)	Dilute with ACN / H ₂ O (50/50, v/v) to a final volume of (mL)	Concentration NF-171 (ng/mL)	Concentration TZ-1E (ng/mL)	Concentration TZ-2 (ng/mL)	Solution name
ZV1	1.0	20	0.378	0.375	0.753	Cal 1
ZV1	0.677	20	0.252	0.250	0.502	Cal 2
ZV1	0.333	20	0.126	0.125	0.251	Cal 3
ZV1	0.167	20	0.063	0.063	0.126	Cal 4
ZV1	0.067	20	0.025	0.025	0.050	Cal 5
ZV1	0.033	20	0.012	0.012	0.025	Cal 6
ZV1	0.02	20	0.008	0.008	0.015	Cal 7

Concentrations shown are rounded values, while they were calculated with the exact data.

5.5 Calculations

The detector signals were registered and integrated using the data system outlined in chapter 5.3. The peak area was taken into account to determine the amount of NF-171, TZ-1E and TZ-2 in the specimens. The calibration curves were calculated from the area of the calibration solutions with their corresponding concentrations of NF-171, TZ-1E and TZ-2 in using equation (1):

$$(1) \quad y = a + bx$$

where

- y: peak area [integration units iu]
- x: amount of analyte injected [pg]
- a: ordinate intercept [iu]
- b: slope [iu/pg]

The amount of NF-171, TZ-1E and TZ-2 in in the specimens was calculated using the transformed equation (1):

$$(2) \quad x = \frac{y - a}{b}$$

The concentration C of NF-171, TZ-1E and TZ-2 in in the specimens was calculated from x using equation (3):

$$(3) \quad C = \frac{x \cdot V_E}{V_i \cdot V}$$

where

- C : analysed concentration of analyte in the specimen (µg/L)
- x : amount of analyte in the specimen portion [ng]
- V_E : final volume (mL)
- V_i : injection volume (µL)
- V : specimen volume (mL)

The recovery data was calculated according to equation (4):

$$(4) \quad R = \frac{C_R \cdot 100}{C_F}$$

where

- R : recovery [%]
- C_R: analysed concentration of analyte in the fortified specimen (µg/L)
- C_F: nominal concentration of analyte in the fortified specimen (µg/L)

The recovery values reported in the Tables of Chapter 6.8 and tables 4 - 6 represent rounded results which were obtained from calculations based on the exact raw data.

5.6 Example for Calculation

The fortified surface water specimen 170474280-A was extracted and analysed on 15 May 2017. The parameters of the calibration curve for NF-171 on the basis of the detected transition m/z 410 > 310 were $a = -727.5$ (ordinate intercept) and $b = 162674.9$ derived from equation (1). The peak area of the NF-171 analyte corresponded to 18916 counts.

The amount of NF-171 was calculated according to equation (2):

$$x = \frac{18916iu - (-727.5iu)}{162674.9 \frac{iu}{pg}} = 0.1208[pg] = 0.0001208[ng]$$

The residue concentration was calculated according to equation (3):

$$C = \frac{0.0001208ng \cdot 100 mL}{0.005 ml \cdot 50 ml} = 0.0483 \left[\frac{ng}{mL} \right] = 0.0483 \left[\frac{\mu g}{L} \right]$$

where

x:	0.001208 [ng]
C:	analysed concentration of NF-171 [$\mu\text{g/L}$]
V_E :	100 [mL]
V_i :	5 [μL]
V:	50 [mL]

The recovery data were calculated according to equation (4).

$$REC = \frac{0.0483 \cdot 100}{0.0503} = 96.0\% \quad (4)$$

where

REC:	recovery rate [%]
C_{REC} :	0.0483 [$\mu\text{g/L}$]
C_{FOR} :	0.0503 [$\mu\text{g/L}$]

The tabulated values were rounded values based on calculations with the exact raw data.

6.11 Conclusion

The validation of the analytical method developed under non-GLP conditions at SGS INSTITUT FRESENIUS project IF-17/04068807 for the determination of residues of NF-171, TZ-1E and TZ-2 in surface water was successful. NF-171, TZ-1E and TZ-2 were shown to be stable in surface water specimens stored at $\leq -18^{\circ}\text{C}$ for 28 days.

Details about the preparation of the fortified specimens are reported in Table 3.

Detailed results of the fortified specimens are reported in Tables 4 to 6 and detailed results of storage stability specimens are reported in Tables 7 to 10.

A typical calibration curve of NF-171 and typical chromatograms are shown in Figures 1 to 10. A typical calibration curve of TZ-1E and typical chromatograms are shown in Figures 13 to 22. A typical calibration curve of TZ-2 and typical chromatograms are shown in Figures 25 to 34.

Spectra of NF-171 are shown in Figure 11 to 12, for TZ-1E in Figure 23 to 24 and for TZ-2 in Figure 35 to 36.

One person needs 4.5 h for 14 specimens for extraction and evaluation (not taking into account the HPLC run times).

The residue analytical method fulfils the acceptance criteria as defined in the EU Guidance document on Residue Analytical Method (SANCO/825/00 rev. 8.1 16/11/2010).

7 List of References

- [1] Determination of Picarbutrazox (NF-171) and Metabolites (TY-1, TY-2, TZ 1E, TZ 2, TZ-2E, TZ-4, and TZ-5) in Soil using LC-MS/MS, Method No.: 81543-M1
- [2] Method Development for the Analysis of NF-171, TZ-1E and TZ-2 in surface water, Project Number IF-17/04068807 (developed under non-GLP conditions)

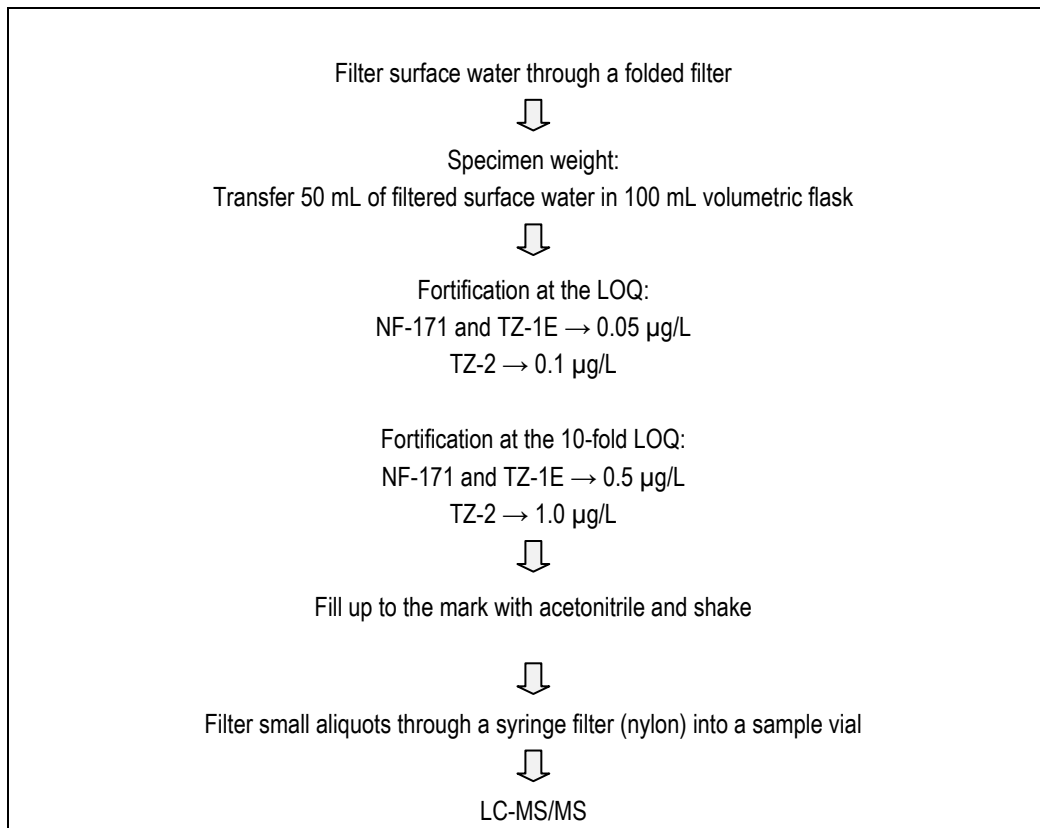


Fig. 37: Flow Chart for the analysis of NF-171, TZ-1E and TZ-2 in water by LC-MS/MS