TITLE

Independent Laboratory Validation of an Analytical Method for the Determination of Picarbutrazox (NF-171) and Metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in Soil

TEST GUIDELINE

US EPA OCSPP 860.1340 and 850.6100

AUTHORS

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STUDY COMPLETION DATE

August 2, 2017

PERFORMING LABORATORY

EAG Laboratories-Hercules
625-B Alfred Nobel Drive
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SPONSOR

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Wall Street Plaza
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New York, NY 10005

LABORATORY PROJECT ID

Project Number: 2912W

SUMMARY

A residue method for the determination of Picarbutrazox (NF-171) and seven metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil system was independently validated. The original method was described in “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", EAG Laboratories-Columbia, Method No. 81543-M1, October 14, 2016.

The method for the analysis of NF-171 and seven metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil samples consisted of extraction with ammonium chloride and methanol followed by another extraction with 75:25 methanol/0.1% formic acid. Supernatants were combined and diluted to 50 mL with methanol; an aliquot of the sample extracts was filtered through a 0.2 µm nylon syringe filter and diluted with methanol and water prior to analyzing with liquid chromatography tandem mass spectrometry (LC-MS/MS) with positive electrospray ionization.

The limit of quantitation (LOQ) was targeted at 0.01 mg/kg (ppm) for all analytes in soil matrix as validated in this study.

The independent laboratory validation (ILV) was conducted with one reagent blank, two untreated soil samples (controls) and five soil samples spiked at each fortification level: one at LOQ level and another at 10X LOQ level.

NF-171 and seven metabolites were quantitated against separate 1/x weighted linear regression curves of the reference substances NF-171, TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5 whose concentrations ranged from 0.1 ng/mL to 5 ng/mL. The calibration for each analyte yielded acceptable correlation coefficient ($r > 0.99$) over the range examined. The quantitation of each analyte was based on the peak area response and concentration of the calibration standards. The amount of NF-171 and metabolites was determined with the corresponding quantitation ion. In addition, confirmation MS/MS ion transition data were collected for all analytes. Recoveries from fortified samples were determined by calculating the found amount of individual compound against the theoretical amount and averaging by the relevant fortification level.

INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of Picarbutrazox (NF-171) and seven metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil. The analysis of the reference/test substances was performed by liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS) based on the method "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", EAG Laboratories-Columbia, Method No. 81543-M1, October 14, 2016, provided by the sponsor.

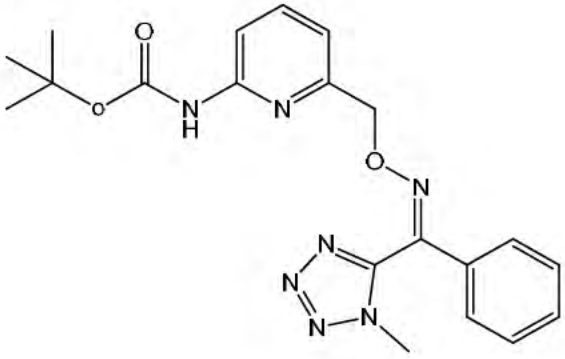
This study was designed to satisfy US EPA Guideline requirements described in OCSPP 860.1340 and 850.6100. The study was initiated on January 6, 2017 at EAG Laboratories-Hercules, 625-B Alfred Nobel Drive, Hercules, CA 94547 under an approved protocol (Appendix A) according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160.

MATERIAL AND METHODS

Test/Reference Substances

Picarbutrazox:

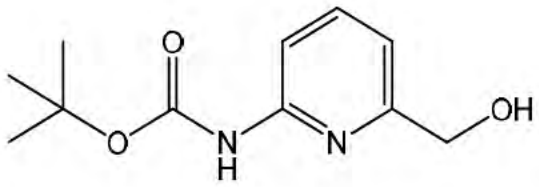
Common Name:	Picarbutrazox
Experimental Name:	NF-171
CAS Name:	1,1-dimethylethyl *N*-[6-[[[(Z)-[(1-methyl-1*H*-tetrazol-5-yl)phenylmethylene]amino]oxy]methyl]-2-pyridinyl]carbamate
IUPAC Name:	*Tert*-butyl (6-{[(*Z*)-(1-methyl-1*H*-5-tetrazolyl)(phenyl)methylene]aminooxymethyl}-2-pyridyl) carbamate
CAS No.:	500207-04-5
Inventory No.:	2912W-001
Molecular Weight:	409.44 g/mol
Molecular Formula:	$C_{20}H_{23}N_7O_3$
Structural Formula:	



TY-1:

Experimental Name:	TY-1
Chemical Name:	*Tert*-butyl [6-(hydroxymethyl)-2-pyridyl]carbamate
CAS No.:	203321-83-9
Inventory No.:	2912W-005
Molecular Weight:	224.26 g/mol
Molecular Formula:	$C_{11}H_{16}N_2O_3$

Structural Formula:



TY-2:

Experimental Name: TY-2

Chemical Name: (6-amino-2-pyridyl)methanol

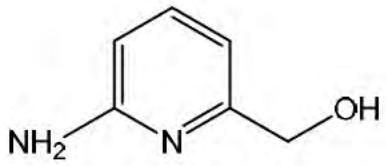
CAS No.: 79651-64-2

Inventory No.: 2912W-008

Molecular Weight: 124.14 g/mol

Molecular Formula: $C_6H_8N_2O$

Structural Formula:



TZ-2:

Experimental Name: TZ-2

Chemical Name: (*Z*)-*O*-[(6-amino-2-pyridyl)methyl](1-methyl-1*H*-5-tetrazolyl)(phenyl)methanone oxime

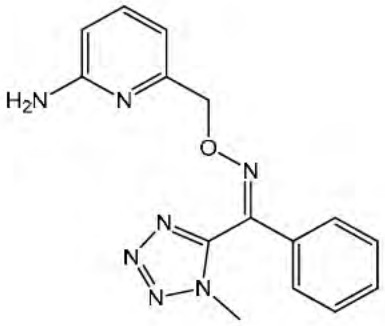
CAS No.: 500206-79-1

Inventory No.: 2912W-007

Molecular Weight: 309.33 g/mol

Molecular Formula: $C_{15}H_{15}N_7O$

Structural Formula:



TZ-1E:

Experimental Name: TZ-1E

Chemical Name: *Tert*-butyl (6-{[(*E*)-(1-methyl-1*H*-5-tetrazolyl)(phenyl)methylene]-aminooxymethyl}-2-pyridyl)carbamate

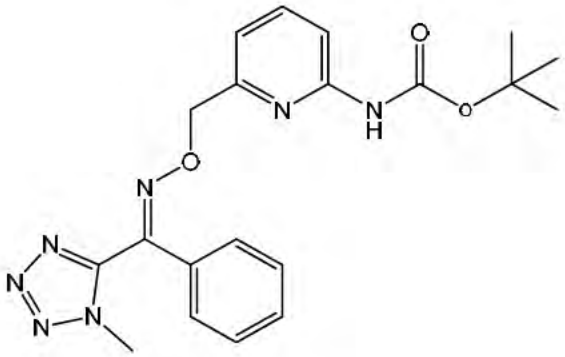
CAS No.: 1253511-94-2

Inventory No.: 2912W-006

Molecular Weight: 409.44 g/mol

Molecular Formula: $C_{20}H_{23}N_7O_3$

Structural Formula:



TZ-2E:

Experimental Name: TZ-2E

Chemical Name: (*E*)-*O*-[(6-amino-2-pyridyl)methyl](1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanone oxime

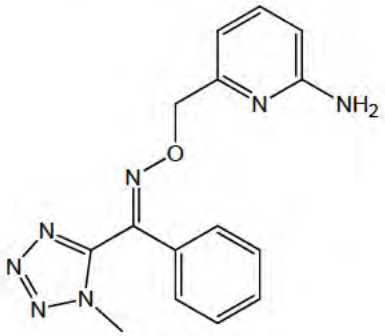
CAS No.: NA

Inventory No.: 2912W-004

Molecular Weight: 309.33 g/mol

Molecular Formula: $C_{15}H_{15}N_7O$

Structural Formula:



TZ-4:

Experimental Name: TZ-4

Chemical Name: (1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanone

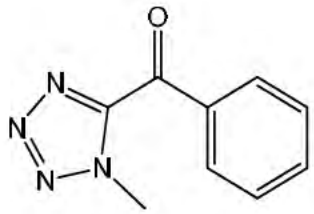
CAS No.: 33452-25-4

Inventory No.: 2912W-003

Molecular Weight: 188.19 g/mol

Molecular Formula: $C_9H_8N_4O$

Structural Formula:



TZ-5:

Experimental Name: TZ-5

Chemical Name: (1-methyl-1*H*-5-tetrazolyl)(phenyl)methanol

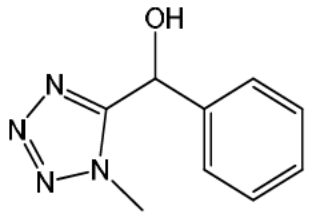
CAS No.: 33452-21-0

Inventory No.: 2912W-002

Molecular Weight: 190.20 g/mol

Molecular Formula: $C_9H_{10}N_4O$

Structural Formula:



Picarbutrazox (NF-171) and seven metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5 were provided by the Sponsor on December 15, 2016. Upon receipt at EAG Laboratories-Hercules, the test/reference substances were assigned the inventory No. 2912W-001 through 2912W-008. The test/reference substances were stored frozen (typically < -4 °C) when not in use.

Inventory no.	Analyte	Lot No.	Purity (%)	expiration date
2912W-001	NF-171	31-09142-Y.MIZOGUCHI	98.8	Aug 30, 2018
2912W-002	TZ-5	31-10172-T.SUGIURA	>99.9	Aug 17, 2018
2912W-003	TZ-4	31-08259-M.IWASAWA	>99.9	Aug 28, 2018
2912W-004	TZ-2E	31-12282-T.SUGIURA	99.7	Oct 4, 2018
2912W-005	TY-1	31-10181-T.SUGIURA	95.4	April 24, 2017
2912W-006	TZ-1E	31-10187-T.SUGIURA	98.0	May 11, 2018
2912W-007	TZ-2	31-10242-T.SUGIURA	99.7	March 4, 2018
2912W-008	TY-2	16031	98.9	Dec 1, 2019

The certificates of analysis are provided in Appendix B.

Reagents

HPLC grade water, methanol, acetone, acetonitrile (ACN), were obtained from Fisher Chemical; ammonium hydroxide (30%) and formic acid was obtained from Fisher Chemical; ammonium chloride was obtained from Sigma Aldrich and Fisher Scientific.

Equipment/Materials List

Laboratory Balances
Geno/Grinder 2010
Stainless steel grinding balls (SPEX Sample Prep, 4mm. Part No. 2150)
Pipettes
Centrifuge
Volumetric flasks
Centrifuge tubes (50 mL capacity)
Vortex
Variable volume pipetors with plastic disposable tips
Hamilton glass precision syringes
0.1 µm Millipore Durapore® membrane filter with vacuum filter apparatus
0.2 µm nylon syringe filters
Amber bottles and vials with Teflon® lined caps

AB Sciex API 5500 Series Triple Quad Mass Spectrometer with Agilent 1260 Series LC (LC-MS/MS), Analyst Data System Software

Test System

Source of the Test System

A sandy loam/loam soil was shipped to EAG Laboratories-Hercules from EAG Laboratories-Columbia (7200 East ABC Lane, Columbia, MO 65202) and given the inventory No. 2912W-009. The test system was stored frozen (typically < -4°C) in the dark when not in use.

Characterization of the Test System

The soil used in the study was characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). The characterization report is presented in Appendix C.

Test Method

The analytical method for the analysis of Picarbutrazox (NF-171) and seven metabolites (TY-1, TY-2, TZ-2, TZ-1E, TZ-2E, TZ-4, and TZ-5) in soil was independently validated at EAG Laboratories-Hercules by LC-MS/MS. Analyses of the eight analytes were based on the analytical method described in "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", EAG Laboratories-Columbia, Method No. 81543-M1, October 14, 2016.

The method for the analysis of NF-171 and seven metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil samples consisted of extraction with ammonium chloride and methanol followed by another extraction with 75:25 methanol/0.1% formic acid. Supernatants were combined and diluted to 50 mL with methanol; an aliquot of the sample extracts was filtered through a 0.2 µm nylon syringe and diluted with methanol and water filter prior to analyzing with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The percent recovery of the analytes was determined using external standardization where separate linear curves of calibration standards for each analyte were analyzed along with the samples.

Preparation of Stock Solutions

Separate stock solutions of NF-171 and metabolites (TY-1, TY-2, TZ-2, TZ-1E, TZ-2E, TZ-4, and TZ-5) were prepared by weighing aliquots (approximately 10 mg) of the test/reference substances in weighing boats, transferring to volumetric flasks with some ACN and sonicating for approximately 30 seconds. Final solutions were diluted to the mark with ACN. Additional solvent was added as necessary to achieve a nominal concentration of 1.0 mg/mL after adjusting for the purity of the reference substances as follows:

Analyte	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical conc. (µg/mL) [1]
NF-171	11.37	11.23	98.8	1,000
TZ-5	10.63	10.62	99.9	1,000
TZ-4	10.78	10.75	99.9	1,002
TZ-2E	10.43	10.38	99.7	1,002
TY-1	11.70	11.16	95.4	1,000
TZ-1E	10.29	10.08	98.0	1,000
TZ-2	10.42	10.38	99.7	1,001
TY-2	10.21	10.09	98.9	1,001

[1]Theoretical conc. (µg/mL) = [weight (mg) x 1,000 µg/mg ÷ final volume (mL)] x [purity (%)]
Note: purity of TZ-5 and TZ-4 was > 99.9%; used 99.9% as worst case scenario.

The stock solutions were transferred into an amber bottle and stored refrigerated (typically < 4°C) when not in use. As verified in the analytical method "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", these stock standard solutions are stable for at least 421 days (296 days for TY-1 and TY-2).

The stock solutions were prepared in January 2017 and used throughout this study. In particular, eight stock solutions were used to prepare fresh fortification and other solutions in March 2017 for ILV#1; TY-2 stock solution was used to prepare fresh fortification and other solutions in May 2017 for ILV#2.

Preparation of Fortification Solutions

For ILV#1, a high fortification solution containing a mixture of NF-171, TY-1, TY-2, TZ-2, TZ-1E, TZ-2E, TZ-4, and TZ-5 was prepared at 5.0 µg/mL by measuring an aliquot (0.05 mL) of each stock solution (1.0 mg/mL) and transferring into a 10 mL volumetric flask. For ILV#2, a high fortification solution containing only TY-2 was prepared at 5.0 µg/mL by measuring an aliquot (0.05 mL) of TY-2 stock solution (1.0 mg/mL) and transferring into a 10 mL volumetric flask. Solution was diluted to the mark with ACN and mixed.

For ILV#1, a low fortification solution a mixture of NF-171 and metabolites was prepared at 0.5 µg/mL by measuring an aliquot (1.0 mL) of the 5.0 µg/mL high fortification solution and transferring into a 10 mL volumetric flask. For ILV#2, a low

fortification solution only TY-2 was prepared at 0.5 µg/mL by measuring an aliquot (1.0 mL) of the 5.0 µg/mL high fortification solution and transferring into a 10 mL volumetric flask. Solution was diluted to the mark with ACN and mixed.

Solution used	Aliquot (mL)	Final volume (mL)	Theoretical conc. (µg/mL) [1]	Sample ID
Individual stock (1.0 mg/mL)	Each 0.05	10	5.0	5.0 µg/mL High fortification solution
High Fort (5.0 µg/mL)	1.0	10	0.5	0.5 µg/mL Low fortification solution

[1]Theoretical conc. (µg/mL) = {[theoretical conc. solution used x aliquot (mL)] ÷ final volume (mL)}

Fortification solutions were stored refrigerated (typically < 4°C) when not in use. As verified in the analytical method "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", these fortification standard solutions are stable for at least 29 days.

The fortification solutions used for each ILV were freshly prepared from stock standard solutions and used within a week of preparation.

Preparation of Intermediate Solution

For ILV#1, an intermediate solution containing NF-171 and metabolites was prepared by measuring an aliquot (0.8 mL) of the 5.0 µg/mL high fortification solution into a 20 mL volumetric flask. Solution was diluted to the mark with methanol to yield a nominal assay concentration of 200 ng/mL and mixed. For ILV#2, an intermediate solution containing only TY-2 was prepared by measuring an aliquot (0.8 mL) of the 5.0 µg/mL high fortification solution into a 20 mL volumetric flask. Solution was diluted to the mark with methanol to yield a nominal assay concentration of 200 ng/mL and mixed. The intermediate solution was stored refrigerated (typically < 4°C) when not in use. As verified in the analytical method "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", the intermediate solution is stable for at least 11 days.

The intermediate solution used for each ILV was freshly prepared from fortification solutions and used within a week of preparation.

Preparation of Intermediate Calibration Standard Solutions

Seven intermediate calibration standard solutions containing either NF-171 and metabolites (for ILV#1) or TY-2 only (for ILV#2) were prepared by measuring an appropriate volume of the 5.0 µg/mL high fortification solution and/or the 200 ng/mL intermediate solution and transferring into separate 10 mL volumetric flasks. Solutions were diluted to the mark with methanol. The concentration of NF-171 and analytes ranged from 2.0 ng/mL to 100 ng/mL as shown below:

Aliquot (mL)	Solution used	Final volume (mL)	Theoretical conc. [1] (ng/mL) Analyte
0.100	200 ng/mL	10.0	2.0
0.250	200 ng/mL	10.0	5.0
0.500	200 ng/mL	10.0	10.0
0.750	200 ng/mL	10.0	15.0
1.000	200 ng/mL	10.0	20.0
2.500	200 ng/mL	10.0	50.0
5.000	200 ng/mL	10.0	100.0

[1]Theoretical conc. (ng/mL) = {[theoretical conc. solution used (ng/mL) x aliquot (mL)] ÷ final volume (mL)}

The intermediate calibration standard solutions were stored refrigerated (typically < 4°C) when not in use. As verified in the analytical method “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", the intermediate calibration standard solutions are stable for at least 25 days when stored refrigerated.

The intermediate calibration standard solutions used for each ILV were freshly prepared from intermediate solution and used within a week of preparation.

Preparation of Matrix-Based Calibration Standard Solutions

Eight calibration standard solutions containing either NF-171 and metabolites (for ILV#1) or TY-2 only (for ILV#2) were prepared in soil matrix by combining an aliquot (0.025 mL) of the corresponding intermediate standard solution with an aliquot (0.475 mL) of control (untreated soil sample) in autosampler vials. The concentration of individual analyte ranged from 0.1 ng/mL to 10 ng/mL; however calibration standard solutions used for calibration was only in the range from 0.1 ng/mL to 5 ng/mL.

Aliquot (mL)	Solution used	Control aliquot (mL)	Final volume [1] (mL)	Theoretical conc. [2] (ng/mL) Analyte
0.025	200 ng/mL	0.475	0.500	10.00
0.025	2 ng/mL	0.475	0.500	0.10
0.025	5 ng/mL	0.475	0.500	0.25
0.025	10 ng/mL	0.475	0.500	0.50
0.025	15 ng/mL	0.475	0.500	0.75
0.025	20 ng/mL	0.475	0.500	1.00
0.025	50 ng/mL	0.475	0.500	2.50
0.025	100 ng/mL	0.475	0.500	5.00

[1] Final volume (mL) = aliquot + control aliquot

[2] Theoretical conc. (ng/mL) = {[theoretical conc. solution used (ng/mL) x aliquot (mL)] ÷ final volume (mL)}

Note: autosampler vials were previously rinsed with acetone and dried before used.

Matrix-based calibration standard solutions were stored refrigerated (typically < 4°C) when not in use. As verified in the analytical method “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", the matrix-based calibration standard solutions are stable for at least 21 days.

The matrix-based calibration standard solutions used for each ILV were freshly prepared from intermediate calibration solutions and used within a week of preparation.

Fortification Procedure

Fortification of untreated soil samples was conducted at two fortification levels as shown below:

Test system (Matrix)	Fortification Level (mg/kg)	Fortification volume (mL)	Solution used
Soil (5 g)	0.01	0.1	0.5 µg/mL Low fortification solution
	0.1	0.1	5.0 µg/mL High fortification solution

Fortification was conducted to determine the percent recovery within the Independent Laboratory Validation. This procedure was performed in quintuplicate during Independent Laboratory Validation at each fortification level.

Extraction Procedure for NF-171 and Seven Metabolites in Soil

1. Weigh soil (5 g) into a centrifuge tube (50 mL).
2. Fortify the samples as needed.
3. Add two stainless steel grinding balls (SPEX Sample Prep, 4mm. Part No. 2150), 1.25 g ammonium chloride and 25 mL methanol.
4. Extract on Geno/Grinder 2010 at 1,200 RPM for 5 minutes.
5. Centrifuge samples at 3,000 RPM for 5 minutes.
6. Decant supernatant into a clean 50 mL centrifuge tube.
7. Add 15 mL 75:25 (v/v) methanol/0.1% formic acid to soil and extract at 1,200 RPM for 5 minutes.
8. Centrifuge samples at 3,000 RPM for 5 minutes.
9. Decant supernatant into the centrifuge tube containing the first extract to combine.
10. Dilute combined extract to final volume of 50 mL with methanol and vortexed to mix.
11. Filter ~ 5 mL of the combined extract through a 0.2 µm nylon syringe filter into a 15 mL plastic tube.
12. Combine 1 mL filtered extract with 1 mL methanol and 2 mL HPLC water and mix in the 15 mL plastic tube.
13. Aliquot final extracts in autosampler vials and analyze by LC-MS/MS.

LC-MS/MS Parameters for ILV#1

LC conditions

Column: Phenomenex Kinetex 2.6µm (50 mm x 2.1 mm) outfitted with a 0.5 µm pore size stainless steel frit attached pre-column
Column Temperature: 40°C
Injection Volume: 10 µL
Mobile Phase: A) 0.01% ammonium hydroxide in HPLC Water
B) 100% ACN
Gradient Program:

Time (min.)	Flow Rate (µL/min)	% A	% B
0.00	800	98	2
0.30	800	98	2
4.00	800	10	90
5.00	800	10	90
5.01	800	98	2
6.00	800	98	2

Approximate retention times:

- TY-2: 0.5-0.6 minutes
- TZ-5: 1.77 minutes
- TY-1 2.38 minutes
- TZ-4: 2.45 minutes
- TZ-2E: 2.54 minutes
- TZ-2: 2.66 minutes
- TZ-1E: 3.34 minutes
- NF-171: 3.41 minutes

MS conditions

Electrospray Ionization (ESI) in positive polarity mode

Acquisition scan mode: Multiple Reaction Monitoring (MRM)

Period 1 settings Experiment 1

For quantitation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
NF-171	410	310	50	20	25	25
TY-1	225	169	100	15	20	25
TY-2	125	107	150	18	13	25
TZ-1E	410	310	45	20	13	25
TZ-2	310	123	10	30	15	25
TZ-2E	310	107	10	25	15	25
TZ-4	189	105	50	19	15	25
TZ-5	191	145	130	15	12	25

For confirmation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
NF-171	410	107	50	32	10	25
TY-1	225	107	100	30	10	25
TY-2	125	80	150	27	14	25
TZ-1E	410	107	45	35	10	25
TZ-2	310	107	10	35	15	25
TZ-2E	310	80	10	57	15	25
TZ-4	189	77	50	45	15	25
TZ-5	191	117	130	21	13	25

	Period 1 Experiment 1
CUR:	40.0
CAD:	9.0
IS:	3000.0
TEM:	750.0
GS1:	50.0
GS2:	70.0
EP:	6.0

LC-MS/MS Parameters for ILV#2

LC conditions

Column: Phenomenex Kinetex 2.6µm (50 mm x 2.1 mm) outfitted with a 0.5 µm pore size stainless steel frit attached pre-column
Column Temperature: 40°C
Injection Volume: 6 µL
Mobile Phase: A) 0.01% ammonium hydroxide in HPLC Water
B) 100% ACN
Gradient Program:

Time (min.)	Flow Rate (µL/min)	% A	% B
0.00	700	98	2
0.30	700	98	2
4.00	700	10	90
5.00	700	10	90
5.01	700	98	2
6.00	700	98	2

Approximate retention times:

- TY-2: 0.5 minutes

MS conditions

Electrospray Ionization (ESI) in positive polarity mode

Acquisition scan mode: Multiple Reaction Monitoring (MRM)

Period 1 settings Experiment 1

For quantitation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
TY-2	125	107	70	18	13	25

For confirmation:

Compound name	Molecular ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Dwell (msec)
TY-2	125	80	70	27	14	25

	Period 1 Experiment 1
CUR:	40.0
CAD:	9.0
IS:	3000.0
TEM:	750.0
GS1:	50.0
GS2:	70.0
EP:	8.0

LC-MS/MS Analysis

For LC-MS analyses, samples were analyzed interspersed among the matrix-based calibration standard solutions in single injection. Two matrix-based calibration standard solutions were reanalyzed as quality control standard (QC) at the end of the sequence. Methanol: water (25:75) was analyzed as the solvent blank.

The stability of the signal was monitored by comparing the response (analyte peak area) of a quality control standard injection with that of a comparable standard from the linear curve of the corresponding analyte within the sequence.

Methods of Calculation

Quantitation

NF-171, TY-1, TY-2, TZ-2, TZ-1E, TZ-2E, TZ-4, and TZ-5 were quantitated by the external standard method using a seven-point linear curve regression for each analyte. Separation of the analytes was achieved by LC-MS/MS in MRM mode. The compounds were identified by the coincidence of their retention times with their respective reference standards and MS characteristics. The quantitation of NF-171 and metabolites was conducted by peak area of each compound relative to the theoretical concentration of the calibration standard solutions. The content of each compound in soil samples except was quantitated against separate 1/x weighted linear curves ($y = mx + b$) of NF-171 TY-1, TY-2, TZ-2, TZ-1E, TZ-2E, TZ-4, and TZ-5 calibration standards where:

y = peak area
x = ng/mL compound injected
m = slope
b = intercept

Weighting of the calibration curve of each analyte was applied so as to provide better curve fit at the lower concentration levels of each analyte. The calculation of weighted curve equations (linear regression) and concentration (ng/mL) present in samples and calibration standards was conducted using Analyst® software.

Recoveries from samples were determined by averaging the found amount recovered (µg) of the analytes (corrected for mean control contribution, if necessary) and dividing by the relevant theoretical fortified amount (µg).

Residue in soil

% Recovery (%) = [(µg recovered – µg mean control) ÷ µg fortified)] x 100

Where:
µg recovered = [calculated concentration (ng/mL) ÷ 1,000 ng/µg] x dilution factor x final volume (mL)
Dilution factor = 4 mL ÷ 1 mL aliquot sample extract
µg fortified = sample weight (g) x fortification level (µg/g)
Calculated concentration (ng/mL) was determined by Analyst® software

LOQ theoretical/expected concentration (ng/mL) in soil samples

LOQ (ng/mL) = (µg fortified x 1000 ng/µg ÷ final volume mL) ÷ dilution factor
Where:
µg fortified = sample weight (g) x fortification level (µg/g)

Example:
Analyte = NF-171
Fortification level (µg/g) = 0.01 (equivalent to 0.01 mg/kg)
Sample weight (g) = 5
Final volume (mL) = 50
Dilution factor = 4

NF-171 fortified (µg) = 5 g x 0.01 µg/g = 0.05
LOQ (ng/mL) = [(0.05 µg x 1000 ng/µg) ÷ 50 mL)] ÷ 4 = 0.25 ng/mL

Calibration Range

The calibration curves were generated by Analyst® software for each compound in each validation. The calibration range for NF-171 and seven analytes in soil was from 0.1 ng/mL to 5 ng/mL.

Limit of Quantitation

The limit of quantitation (LOQ) was set at 0.01 mg/kg (ppm) for NF-171 and metabolites in soil as validated in this study. The LOQ for all compounds in soil represented 0.25 ng/mL in the respective compound standard solution using the current methodology.

Limit of Detection

The limit of detection (LOD) was defined as approximately 30% of LOQ. The calibration solutions were analyzed to confirm the desired LOD.

The confirmed LOD in soil was 0.003 mg/kg (ppm) for NF-171 and seven metabolites. The LOD for all compounds in soil represented 0.1 ng/mL in the respective compound standard solution using the current methodology.

Time Required for Completion of a Sample Set

A sample set can be completed in one set for efficient handling for each matrix. Each set consisted of a reagent blank, two controls (untreated samples) and five fortified samples at each level (LOQ and 10X LOQ).

Time required for one set from initiation of extraction until the completion of instrumental analysis and data evaluation for soil matrix is as follows:

- Preparation of standard solutions takes approximately 4 hours
- Sample preparation and LC-MS/MS analysis for other metabolites take approximately 4 hours
- Data processing for LC-MS takes approximately 4 hours

TOTAL = approximately 12 hours (1.5 calendar days) for one analyst to complete a sample set to satisfy the validation requirements for soil matrix.

Statistical Methods

Means, standard deviation, relative standard deviation, and 1/x linear regression were the only statistical methods employed in this study.

Communication Pertaining to Independent Laboratory Validation

The instrumentation used in the current study is different from the instrumentation described in "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", EAG Laboratories-Columbia, Method No. 81543-M1, October 14, 2016. Not all the instrument parameters were recorded in the original method report.

On March 10, 2017, the study director requested through the study monitor the complete information of instrument parameters used in the Method No. 81543-M1. The method printout from Analyst® was obtained.

On March 29, 2017, the sponsor accepted results for ILV#1, but expressed the concern about the peak shape of TY-2 even though the validation results were acceptable.

On April 24, 2017, the sponsor requested to conduct ILV#2 for TY-2 only. The purpose was to improve the peak shape caused by the increased injection volume used in ILV#1 while still achieving acceptable recovery.

On June 20, 2017, the sponsor accepted validation results for ILV#2.

Modifications of the Original Analytical Method

Due to the difference in the instrumentation used in the original method validation study and the current study, minor modifications were employed during conduct of the independent laboratory validation.

Agilent 1260 LC system used in the current study has noticeably larger dwell volume than Waters UPLC system used in the original method validation. During ILV#1, mobile

phase flow rate was changed from 0.7 mL/min to 0.8 mL/min. It was necessary to compensate the difference in dwell volumes of two types of LC systems for most of the analytes. During ILV#2, the flow rate of 0.7 mL/min was used for TY-2 since the retention time of TY-2 was only about 0.5 minutes.

The original method validation utilized AB Sciex API 5500 LC-MS/MS with Q-trap, while the current study utilized AB Sciex API 5500 without Q-trap. While the sensitivity of the original method was optimized with Q-trap setup, during ILV#1 the following modifications were found necessary to achieve the desired sensitivity for most of the analytes:

1. The injection volume was increased from 5 µL to 10 µL. It was partly made possible with the increased mobile phase flow rate.

2. Q3 resolution was changed from unit to low.

3. Source temperature was changed from 450°C to 750°C to help in the ionization of the earlier eluting compounds.

During ILV#2, the injection volume was changed to 6 µL to achieve an improvement of the peak shape of TY-2.