STUDY TITLE

Method Validation of Picarbutrazox (NF-171) and Metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in Soil and NF-171 and Metabolites (TY-2, TZ-1E, TZ-5, TZ-2-β-Glc and TZ-5-Glc) in Grass Clippings using LC-MS/MS

TEST GUIDELINES

U.S. EPA Residue Chemistry Test Guidelines, January 2012, OCSPP 850.6100, 860.1340 "Environmental Chemistry Methods and Associated Independent Laboratory Validation"

European Commission, SANCO/825/00 rev. 8.1
Guidance Document on Pesticide Residue Analytical Methods

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SUMMARY

These analytical methods were developed and validated for the detection and quantitation of picarbutrazox (NF-171) and its metabolites [TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5] in soil, and NF-171 and its metabolites [TY-2, TZ-1E, TZ-5, TZ-2-β-Glc and TZ-5-Glc] in grass clippings. The method limit of quantitation (LOQ) in soil and grass clippings was 0.01 ppm (mg/kg). The limit of detection (LOD) was defined as approximately 1/3 LOQ or 0.003 ppm (mg/kg) in soil and grass clippings. The methods were validated at 0.01 and 0.10 ppm in soil and grass clippings using an LC-MS/MS system in positive ion mode. The analytical methods are suitable for enforcement/monitoring and data generation for regulatory studies. The method was validated on New York and Idaho soil, and Idaho grass clippings transferred from an ongoing Isagro S.p.A. terrestrial field dissipation study, American Agricultural Services Study No. AA140717, EAG Study No. 81547.

Residues of picarbutrazox (NF-171) and metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) are extracted from 5.0-g soil samples with 1.25 g ammonium chloride and 25 mL of methanol in the presence of two ¼-inch steel balls on a Geno/grinder® followed by centrifugation. The supernatant from this extraction is filtered (0.2-µm Nylon syringe filter) into a clean mixing cylinder and the pellet re-extracted with 15 mL of 75:25, methanol/0.1% formic acid. This extract is filtered (0.2-µm Nylon syringe filter) and combined with the first extract, then diluted to 50 mL with methanol. An aliquot of the extract is diluted with ultra-pure water before analyzing via liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS).

Residues of picarbutrazox (NF-171) and metabolites (TY-2, TZ-1E, TZ-5, TZ-2-β-Glc, and TZ-5-Glc) are extracted from 2.5-g grass clipping samples wetted with 10 mL of water and allowed to sit for 30 minutes before adding 25 mL of acetonitrile in the presence of two ¼-inch steel balls on a Geno/grinder® followed by centrifugation. The supernatant from the extraction is decanted into a clean mixing cylinder. The pellet is then re-extracted with 25 mL of 70:30 methanol:water and the supernatant added to the mixing cylinder, and the final volume is adjusted to 60 mL with acetonitrile. The extract is purified using a 500-mg Oasis® MCX cation exchange SPE cartridge conditioned with methanol. A 5.0-mL aliquot of sample is loaded onto the cartridge and immediately washed with 5.0 mL of methanol, collecting both eluates in one glass tube that has been calibrated to 10.0 mL ("A" fraction). The remaining analytes are then eluted with 8 mL of 5% ammonium hydroxide in methanol (v/v), which is collected in a separate glass tube that has been calibrated to 10.0 mL ("B" fraction). The sample "A" and "B" fractions are brought to a 10-mL volume with acetonitrile. A 4.0-mL aliquot from each sample (both "A" and "B" fractions) is evaporated to remove the solvent and reconstituted in 1.0 mL of methanol followed by 1.0 mL of water for analysis via liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS).

The confirmatory analysis for the LC-MS/MS method was based on detection of secondary parent-to-daughter ion transitions monitored during the validation. Due to the lack of sensitivity for all other transitions, TZ-5-Glc in grass clippings confirmatory analysis was conducted using the quantitation ion transition under different LC conditions.

1 INTRODUCTION

1.1 Scope

These methods are applicable for the quantitative determination of residues of picarbutrazox (NF-171) and metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil and NF-171 and Metabolites (TY-2, TZ-1E, TZ-5, TZ-2-β-Glc and TZ-5-Glc) in grass clippings. Soil and grass methods were validated over the concentration range of 0.01 to 0.10 ppm with a limit of detection of 0.003 ppm. Common and chemical names, molecular formulas, and the nominal masses for the analytes are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OCSPP 850.6100 (1) and the European Commission Guidance Document on Pesticide Residue Analytical Methods, SANCO/825/00 rev.8.1 (2).

1.2 Method Principle

Soil

Residues of picarbutrazox (NF-171) and metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) are extracted from 5.0-g soil samples with 1.25 g ammonium chloride and 25 mL of methanol in the presence of two ¼-inch steel balls on a Geno/grinder® followed by centrifugation. The supernatant from this extraction is filtered (0.2-µm Nylon syringe filter) into a clean mixing cylinder and the pellet re-extracted with 15 mL of 75:25, methanol/0.1% formic acid. This extract is filtered (0.2-µm Nylon syringe filter) and combined with the first extract, then diluted to 50 mL with methanol. An aliquot of the extract is diluted with ultra-pure water before analyzing via liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS).

Grass Clippings

Residues of picarbutrazox (NF-171) and metabolites (TY-2, TZ-1E, TZ-5, TZ-2-β-Glc, and TZ-5-Glc) are extracted from 2.5-g grass clipping samples wetted with 10 mL of water and allowed to sit for 30 minutes before adding 25 mL of acetonitrile in the presence of two ¼-inch steel balls on a Geno/grinder® followed by centrifugation. The supernatant from the extraction is decanted into a clean mixing cylinder. The pellet is then re-extracted with 25 mL of 70:30 methanol:water and the supernatant added to the mixing cylinder, and the final volume is adjusted to 60 mL with acetonitrile. The extract is purified using a 500-mg Oasis MCX cation exchange SPE cartridge conditioned with methanol. A 5.0-mL aliquot of sample is loaded onto the cartridge and immediately washed with 5.0 mL of methanol, collecting both eluates in one glass tube that has been calibrated to 10.0 mL (“A” fraction). The remaining analytes are then eluted with 8 mL of 5% ammonium hydroxide in methanol, which is collected in a separate glass tube that has been calibrated to 10.0 mL (“B” fraction). The sample “A” and “B” fractions are brought to a 10-mL volume with acetonitrile. A 4.0-mL aliquot from each sample (both “A” and “B” fractions) is evaporated to remove the solvent and reconstituted in 1.0 mL of methanol followed by 1.0 mL of water for analysis via liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS).

1.3 Reference Standards

Standard	Lot Number	Purity	Expiration Date
NF-171	31-09142-Y.MIZOGUCHI	98.8%	30 August 2018
TY-1	31-10181-T.SUGIURA	95.4%	24 April 2017
TY-2	16031	99.5%	25 June 2015
		98.9%	01 December 2019
TZ-1E	31-10187-T.SUGIURA	98.0%	11 May 2018
TZ-2	31-10242-T.SUGIURA	99.8%	03 July 2014
		99.7%	04 March 2018
TZ-2E	31-12282-T.SUGIURA	99.6%	02 January 2016
		99.7%	04 October 2018
TZ-4	31-08259-M.IWASAWA	>99.9%	28 August 2018
TZ-5	31-10172-T.SUGIURA	>99.9%	17 August 2018
TZ-2-β-Glc	31-12273-T.SUGIURA	97.7%	05 September 2016
TZ-5-Glc	CC-166	99.0%	13 February 2016
		99.2%	02 February 2017

The above standards were obtained from the Test Substance Coordinator, LabServices, Hamburg, PA, on the behalf of Nisso America Inc. The certificates of analysis were provided by Nisso Chemical Analysis Service Co., Ltd., and are located in Appendix C.

1.4 Characterization of Control Matrices

Soil and grass clippings were taken from, and details of the GLP characterization results are filed with, AASI Study No. AA140717, EAG Study No. 81547.

1.5 Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, Class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

1.5.1 Laboratory Equipment

Liquid chromatograph, Waters Acquity Column Manager, Waters Acquity Sample Manager, Waters Acquity Binary Solvent Manager, Waters Acquity Sample Organizer

Mass spectrometer, Applied Biosystems/Sciex API 5500 Q-Trap, Applied Biosystems

Mass spectrometer data system, Model Analyst 1.6.2, Applied Biosystems

Column, analytical, Phenomenex Kinetex F5, 2.1 x 50 mm, 2.6 µm

Column, analytical, Phenomenex Kinetex C18, 2.1 mm i.d. × 50 mm, 1.7-µm

1.5.2 Prepared Solutions

The following are typical preparations of solutions and reagents used during the conduct of the study. Volumes may have been adjusted to prepare alternate volumes as necessary.

(25:75) Methanol:Water

Combine 250 mL of methanol with 750 mL of HPLC grade water and mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 3 months when stored at room temperature.

(50:50) Methanol:Water

Combine 500 mL of methanol with 500 mL of HPLC grade water and mix well. The solution is stable for at least 12 months when stored at room temperature.

(70:30) Methanol:Water

Combine 700 mL of methanol with 300 mL of HPLC grade water and mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 12 months when stored at room temperature.

0.01% Ammonium Hydroxide in Water

To a 1-L container, partially filled with 1000 mL HPLC-grade water, add 0.1 mL of 30% ammonium hydroxide and mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 1 week when stored at room temperature.

5% Ammonium Hydroxide in Methanol

To a 500-mL container, partially filled with methanol, add 25 mL of 30% ammonium hydroxide. Bring to a final volume of 500 mL with methanol and mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 3 months when stored at room temperature.

(75:25) Methanol:0.1% Formic Acid

Combine 750 mL of methanol with 250 mL of 0.1% formic acid solution. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 3 months when stored at room temperature.

(2:2:1) Methanol:Isopropanol:Water

To a 1-L bottle, add 400 mL each of methanol and isopropanol plus 200 mL of HPLC-grade water. Mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 1 year when stored at room temperature.

1.0 M Formic acid (aq)

Add 3.82 mL of concentrated formic acid (99%) to 96.18 mL of water and mix well. The solution is stable for at least 12 months when stored at room temperature.

1.0 M Ammonium Formate (aq)

Dissolve 6.31 g of ammonium formate (99%) in 100 mL of water and mix well. The solution is stable for at least 3 months when stored at room temperature.

10 mM Ammonium Formate (aq)

Add 20 mL of 1.0 M ammonium formate (aq) to 2000 mL of water and mix well. The solution is stable for at least 3 months when stored at room temperature.

0.1 mM Formic acid + 0.1 mM Ammonium Formate (aq)

Add 0.400 mL of 1.0 M formic acid (aq) and 0.400 mL of 1.0 M ammonium formate (aq) to 4000 mL of water and mix well. The solution is stable for at least 3 months when stored at room temperature.

0.1% Formic Acid

Add 0.25 mL of concentrated formic acid to 125 mL of HPLC-grade water. Dilute to 250 mL with water. Volumes may be adjusted accordingly for different quantities. The solution is stable for 3 months when stored at room temperature.

(1:1:1) Methanol:Acetonitrile:Water

To a carboy containing 4000 mL of acetonitrile, add 4000 mL of methanol and 4000 mL of HPLC-grade water. Mix well. The solution is stable for at least 12 months when stored at room temperature.

(1:1:2) Methanol:Acetonitrile:Water

To a carboy partially filled with 4000 mL acetonitrile, add 4000 mL of methanol and 8000 mL of HPLC-grade water. Mix well. Volumes were adjusted accordingly for different quantities. The solution is stable for at least 12 months when stored at room temperature.

2 EXPERIMENTAL

2.1 Instrumental Conditions

2.1.1 Typical Liquid Chromatography Operating Conditions

Instrumentation:	MDS SCIEX Q-Trap API 5500 LC-MS/MS System MDS SCIEX Analyst 1.6.2 data system
Columns:	Phenomenex Kinetex F5, 2.1 x 50 mm, 2.6-µm Phenomenex Kinetex C18, 2.1 x 50 mm, 1.7- µm[a]
Column Temperature:	40 °C
Injection Volume:	5 µL
Injection Wash Program:	Autosampler loop and needle washed with: 2:2:1 MeOH:Isopropanol:Water, followed by 1:1:2 MeOH: ACN:Water
Run Time:	6.00 minutes
Mobile Phase:	A: 0.01% ammonium hydroxide (aq) B: acetonitrile A: 10mM ammonium formate (aq)[a] B: methanol[a]
Flow Rate:	0.700 mL/min, no split

[a] Alternate information for LC system used in confirmation of TZ-5-Glc in grass clippings.

Gradient:

Time, min	A, %	B, %
0.00	98	2
0.30	98	2
4.00	10	90
5.00	10	90
5.01	98	2
6.00	98	2

Note: The gradient may be adjusted to obtain satisfactory chromatography on a given system.

2.2 Typical Mass Spectrometry Operating Conditions

Interface:	TIS (turbo ion spray)
Polarity:	Positive (+)
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	Nitrogen @ setting of "40"
Collision Gas (CAD):	Nitrogen @ setting of "High" Nitrogen @ setting of "Medium"[a]
Temperature (TEM):	450°C 500°C[a]
Ion Source Gas 1 (GS1):	60
Ion Source Gas 2 (GS2):	60 40[a]

Period 1	
Pre-acquisition Delay:	0.0 min
Acquisition Time	3.3 min
IonSpray Voltage (IS):	5500 volts
Entrance Potential (EP):	10 volts
Dwell Time (ms):	Soil: 25 Grass Clippings: 15 30[a]

[a] Information for alternate LC system used in confirmation of TZ-5-Glc in grass clippings.

Analytes:	Precursor Ion Q1	Product Ion Q3	Collision Energy (CE)	Declustering Potential (DP)	Cell Exit Potential (CXP)
Picarbutrazox (NF-171)					
(quantitation)	410	310	20 V	63 V	13 V
(confirmation)	410	107	32 V	63 V	13 V
TY-1					
(quantitation)	225	169	15 V	105 V	10 V
(confirmation)	225	107	30 V	105 V	10 V
TY-2					
(quantitation)	125	107	19 V	77 V	14 V
(confirmation)	125	80	28 V	77 V	14 V
TZ-1E					
(quantitation)	410	310	20 V	85 V	20 V
(confirmation)	410	107	35 V	85 V	20 V
TZ-2					
(quantitation)	310	123	30 V	130 V	15 V
(confirmation)	310	107	35 V	130 V	15 V

Analytes:	Precursor Ion Q1	Product Ion Q3	Collision Energy (CE)	Declustering Potential (DP)	Cell Exit Potential (CXP)
TZ-2E					
(quantitation)	310	107	25 V	100 V	19 V
(confirmation)	310	80	57 V	100 V	19 V
TZ-4					
(quantitation)	189	105	22 V	50 V	12 V
(confirmation)	189	77	45 V	50 V	12 V
TZ-5					
(quantitation)	191	145	15 V	45 V	15 V
(confirmation)	191	117	22 V	45 V	15 V
TZ-2-β-Glc					
(quantitation)	472	310	25 V	100 V	12 V
(confirmation)	472	107	39 V	100 V	12 V
TZ-5-Glc					
(quantitation)	353	191	18 V	75 V	22 V
(confirmation)[a]	353	191	18 V	45 V	22 V

[a] No alternate transitions were found with enough sensitivity to quantitate at the given LOQ; therefore, confirmation was determined with the quantitation ion using a separate LC system.

2.3 Preparation of Standard Solutions

Typically, primary standard solutions were prepared as follows. Other concentrations or volumes may have been prepared following the same procedures.

Approximately 10 mg (corrected for purity) of each analytical standard was quantitatively transferred to a 10-mL volumetric flask using acetonitrile. The solutions were brought to volume with acetonitrile to make stock standard solutions of approximately 1.00 mg/mL.

The stock standard solutions in acetonitrile are stable for at least 421 days (296 days for TY-1 and TY-2, and 355 days for TZ-2-β-Glc and TZ-5-Glc) when stored capped at ~5°C.

2.4 Preparation of Fortification Solutions

Typically, fortification solutions were prepared as follows. Other concentrations or volumes may have been prepared following the same procedures.

Soil

The following concentrations of fortification standard solutions were prepared using individual stock standard solutions containing NF-171, TZ-1E, TZ-2, TZ-2E, TY-1, TY-2, TZ-4, and TZ-5. These solutions prepared in acetonitrile are stable for at least 29 days when stored capped at ~5°C.

5.0 µg/mL: Transfer 0.050 mL of each 1.0-mg/mL standard solution to a single 10-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

0.50 µg/mL: Transfer 1.0 mL of a 5.0-µg/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

Grass Clippings

The following concentrations of fortification standard solutions were prepared using individual stock standard solutions containing NF-171, TZ-1E, TY-2, TZ-5, TZ-2-β-Glc, and TZ-5-Glc. These solutions prepared in acetonitrile are stable for at least 41 days when stored capped at ~5°C.

5.0 µg/mL: Transfer 0.050 mL of each 1.0-mg/mL standard solution to a single 10-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

0.50 µg/mL: Transfer 1.0 mL of a 5.0-µg/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in acetonitrile. Mix well.

2.5 Matrix-Matched Calibration Standard Solutions

2.5.1 Soil

Option 1:

Matrix-matched calibration standards were injected interspersed with samples throughout each analytical run. Any effects due to matrix were compensated for in the determination of residues. Matrix-matched standards were prepared by adding 0.25 mL of filtered control extract (prior to bringing to final volume) to enough empty small tubes (example: 16 × 100 glass culture tubes) to cover the number of standards to be prepared. All solvent was removed under a stream of nitrogen at ~40 °C on an N-Evap. Residues were reconstituted in a 1.0-mL aliquot of the appropriate intermediate calibration standard concentration below and sonicated to mix. Stability of intermediate and calibration standard solutions in methanol:water (25:75, v/v) and matrix-matched standards prepared following this procedure was not determined. Successful results were obtained when intermediate solutions were used 11 days after preparation and matrix-matched standards were injected up to 2 days after preparation.

Intermediate Standard Solutions:

50 ng/mL: Transfer 1.0 mL of 0.50-µg/mL standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

Intermediate Calibration Standard Solutions:

0.10 ng/mL: Transfer 0.020 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

0.25 ng/mL: Transfer 0.050 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

0.50 ng/mL: Transfer 0.10 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

0.75 ng/mL: Transfer 0.15 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

1.0 ng/mL: Transfer 0.20 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

2.5 ng/mL: Transfer 0.50 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

5.0 ng/mL: Transfer 1.0 mL of 50-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol:water (25:75, v/v). Mix well.

Note: Injection vial must be rinsed with acetone and dried before use. Residues from their manufacture can cause enhanced responses for some analytes.

Option 2:

The following procedure for preparation of matrix-matched standards was developed after validation of the analytical method and proved to be suitable for use. Matrix-matched standards were prepared by diluting the intermediate calibration standards 20X with final control sample extract (matrix suspended in 50:50 methanol:water). For example, 0.025 mL of the intermediate calibration standard solution was added to 0.475 mL of final control sample extract; this resulted in standards ranging from 0.10 to 5.0 ng/mL. Intermediate calibration standard solutions in methanol are stable for at least 25 days when stored capped at ~5 °C. Matrix-matched calibrations standards are stable for at least 21 days when stored capped at ~5 °C.

Intermediate Standard Solutions:

200 ng/mL: Transfer 0.80 mL of 5.0-µg/mL mixed standard solution to a 20-mL volumetric flask. Bring to volume in methanol. Mix well.

Intermediate Calibration Standard Solutions:

2.0 ng/mL: Transfer 0.050 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

5.0 ng/mL: Transfer 0.125 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

10 ng/mL: Transfer 0.250 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

15 ng/mL: Transfer 0.375 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

20 ng/mL: Transfer 0.500 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

50 ng/mL: Transfer 1.25 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

100 ng/mL: Transfer 2.50 mL of 200-ng/mL mixed standard solution to a 5-mL volumetric flask. Bring to volume in methanol. Mix well.

Note: Injection vial must be rinsed with acetone and dried before use. Residues from their manufacture can cause enhanced responses for some analytes.

2.5.2 Grass Clippings

Matrix-matched calibration standards were injected interspersed with samples throughout each analytical run. Any effects due to matrix were compensated for in the determination of residues. Matrix-matched standards were prepared by adding 2.0 mL of cleaned control extract to enough empty glass tubes to cover the number of standards to be prepared. All solvent was removed under a stream of nitrogen at ~40 °C on an N-evap. Acetonitrile was added as needed to remove all water. Residues were reconstituted in a 0.5-mL aliquot of the appropriate intermediate calibration standard concentration below, vortexed and sonicated to dissolve all residues, and 0.50 mL of water was added. These intermediate calibration standard solutions prepared in methanol are stable for at least 45 days when stored capped at ~5 °C. Stability of matrix-matched standards prepared following this procedure was not determined. Successful results were obtained when matrix-matched standards were injected up to 1 day after preparation. It is recommended that matrix-matched standards be prepared fresh for each analysis.

Intermediate Standard Solution:

50 ng/mL: Transfer 0.20 mL of a 5.0-µg/mL mixed standard solution to a 20-mL volumetric flask. Bring to volume in methanol. Mix well.

<u>Intermediate Calibration Standard Solutions:</u>

0.20 ng/mL: Transfer 0.040 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

0.50 ng/mL: Transfer 0.10 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

1.0 ng/mL: Transfer 0.20 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

1.5 ng/mL: Transfer 0.30 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

2.0 ng/mL: Transfer 0.40 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

5.0 ng/mL: Transfer 1.0 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

10 ng/mL: Transfer 2.0 mL of 50.0-ng/mL mixed standard solution to a 10-mL volumetric flask. Bring to volume in methanol. Mix well.

Note: Injection vial must be rinsed with acetone and dried before use. Residues from their manufacture can cause enhanced responses for some analytes.

2.6 Sample Origin, Numbering, Preparation and Storage

Untreated control soil and grass clipping samples were transferred from the New York (soil only) and Idaho trials of an ongoing Isagro S.p.A. terrestrial field dissipation study, under American Agricultural Services Study No. AA140717, EAG Study No. 81547. Chain of Custody documentation is included in the raw data.

Sample preparation was performed by EAG Laboratories. Soil and grass clipping samples were ground using a Hammermill and Robot Coupe grinder, respectively. Dry ice was passed through to cool it before sample processing. Each frozen composite sample was then ground in the presence of enough dry ice to keep the sample frozen.

After grinding, the samples were placed in pre-labeled containers and the dry ice was allowed to sublime in a freezer over several days. The sample grinding equipment was cleaned after each sample was processed to avoid contamination.

The control soil and grass clipping samples from both New York and Idaho were processed and stored frozen prior to analysis.

2.7 Sample Analysis

2.7.1 Soil

Each 5.0-g sample was weighed into a 50-mL polypropylene tube. Recovery samples were made by adding appropriate aliquots of mixed standard solution to obtain concentrations of 0.01 and 0.10 ppm for each analyte as detailed in the table below. (The percent recovery found was calculated for each analyte.)

Sample Description	Fortification Volume (mL)	Mixed Fortification Solution (µg/mL)	Fortification Level (ppm)
CONTROL	---	---	---
LOQ	0.100	0.50	0.01
10 x LOQ	0.100	5.00	0.10

To each sample, 1.25 g of ammonium chloride and 25 mL of methanol, along with two ¼" steel balls were added before shaking on a Geno/grinder® for ~5 minutes at ~1200 rpm and centrifuging for ~5 minutes at ~3000 rpm. The supernatant from this extraction was filtered (0.2-µm Nylon syringe filter) into a clean mixing cylinder, and the pellet re-extracted with 15 mL of 75:25, methanol:0.1% formic acid, shaking again on a Geno/grinder® for ~5 minutes at ~1200 rpm, and centrifuging for ~5 minutes at ~3000 rpm. The supernatant was filtered using a 0.2-µm Nylon filter, combined with the first extract, and brought to a final volume of 50 mL with methanol.

A 1.0-mL aliquot was removed from the extract, diluted to 4.0 mL with ultra-pure water, and vialed for analysis via LC-MS/MS. Matrix matched calibration standards were injected interspersed with samples throughout the run.

2.7.2 Grass Clippings

Each 2.5-g sample was weighed into a 50-mL polypropylene tube. Recovery samples were made by adding appropriate aliquots of mixed fortification solution to obtain concentrations of 0.01 and 0.10 ppm for each analyte as detailed in the table below. (The percent recovery found was calculated for each analyte.)

Sample Description	Fortification Volume (mL)	Mixed Fortification Solution (µg/mL)	Fortification Level (ppm)
CONTROL	---	---	---
LOQ	0.050	0.50	0.01
10 x LOQ	0.050	5.00	0.10

To each sample, 10 mL of water were added and allowed to sit for 30 minutes before adding two ¼" steel balls and 25 mL of acetonitrile, and shaking on a Geno/grinder® for ~3 minutes at ~1200 rpm then centrifuging for ~3 minutes at ~3500 rpm. The supernatant from the extraction was decanted into a clean mixing cylinder and the pellet re-extracted with 25 mL of 70:30 methanol:water, shaking again on a Geno/grinder® for ~3 minutes at ~1200 rpm, and centrifuging for ~3 minutes at ~3500 rpm. The supernatant was combined with the first extract, and brought to a final volume of 60 mL with acetonitrile.

The extracts were purified using 500-mg Oasis® MCX cation exchange SPE cartridges conditioned with methanol. A 5.0-mL aliquot of sample was loaded onto the cartridge and immediately washed with 5.0 mL of methanol, collecting both eluates in one glass tube that had been calibrated to 10.0 mL ("A" fraction). The remaining analytes were then eluted with 8 mL of 5% ammonium hydroxide in methanol, which is collected in a separate glass tube that had been calibrated to 10.0 mL ("B" fraction). The sample "A" and "B" fractions were brought to a 10-mL volume with acetonitrile. A 4.0-mL aliquot from each sample (both "A" and "B" fractions) was evaporated to remove the solvent and reconstituted in 1.0 mL of methanol followed by 1.0 mL of water for analysis via LC-MS/MS. Matrix-matched calibration standards were injected interspersed with samples throughout the run.

2.8 Calculations

Calculations for instrumental analysis were conducted using a validated software application (Applied BioSystems/MDS Sciex Analyst, version 1.6.2) to create a standard curve based on linear regression. The regression functions are used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak area response) and to determine concentrations of the analytes found during sample analysis from the calculated best-fit line. For each analytical batch, matrix matched calibration standards were injected over the linear range of the instrument (typically 0.10 to 5.0 ng/mL). All standards injected and their corresponding peak responses were entered into the program to create the standard curve. Weighting (1/x) was used.
The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = detector response (peak area) for each analyte

X = analyte concentration in the sample in ng/mL

$$X = \frac{Y - \text{intercept}}{\text{Slope}} = \text{ng/mL}$$

The standard (calibration) curve generated for each analytical set was used for the quantitation of Picarbutrazox (NF-171) or metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, TZ-5, TZ-2-β-Glc, and TZ-5-Glc) in the samples from the set. Correlation coefficient (r) for each calibration curve were greater than 0.990 (r^2 equal to or greater than 0.98). The sample results in ng/mL from Analyst were subsequently transferred to Microsoft Excel® and sample concentrations calculated with full precision. There may be slight differences in the calculated results below due to rounding.

For the determination of Picarbutrazox (NF-171) or metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, and TZ-5) in soil (in terms of ppm), the following equation is used:

$$\text{Found (ppm)} = \frac{\text{ng/mL found x Extract Vol. (mL)} \times \text{Final Vol. (mL)} \times \text{DF}}{\text{Aliquot Vol. (mL) x Sample Weight (g)} \times 1000\ \text{ng/µg}}$$

where:

ng/mL found	=	(Peak Area – y-intercept) / slope
Extract Vol. (mL)	=	50
Final Vol. (mL)	=	4.0
DF	=	Dilution factor
Aliquot Vol. (mL)	=	1.0
1000 ng/µg	=	Conversion factor

Sample Weight (g) = 5.0

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{\text{ppm found - ppm found in control}}{\text{ppm added}} \times 100$$

Note: Residue contribution of the control sample below 30% of the LOQ was not subtracted from the fortification residues.

Example: NF-171 recovery of soil sample 81543-MV-70, Fortified Control @ 0.01 ppm, Set #MV-6. See Appendix D.

The concentration determined from the standard curve is = 0.2547 ng/mL.

The residue of NF-171 in the final solution is calculated as follows:

$$\text{NF-171 (ppm)} = \frac{0.2547\ \text{ng/mL} \times 50\ \text{mL} \times 4.0\ \text{mL} \times 1}{1.0\ \text{mL} \times 5.01\ \text{g} \times 1000\ \text{ng/µg}} = 0.01017\ \text{ppm}$$

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{\text{ppm found - ppm (> LOD) found in control}}{\text{ppm added}} \times 100$$

$$\text{Percentage Recovery} = \frac{0.01017\ \text{ppm} - 0.00\ \text{ppm}}{0.01\ \text{ppm}} \times 100 = 102\%\ \text{NF-171 recovery}$$

For the determination of Picarbutrazox (NF-171) or metabolites (TY-2, TZ-1E, TZ-5, TZ-2-β-Glc, and TZ-5-Glc) in grass clippings (in terms of ppm), the following equation is used:

$$\text{Found (ppm)} = \frac{\text{ng/mL found x Extract Vol. (mL) x Post SPE Vol. (mL)} \times \text{Final Vol. (mL)} \times \text{DF}}{\text{Aliquot Vol. (mL) x Post SPE Aliquot Vol. (mL) x Sample Weight (g)} \times 1000\ \text{ng/µg}}$$

where:

ng/mL found = (Peak Area – y-intercept) / slope

Extract Vol. (mL) = 60

Aliquot Vol. (mL) = 5.0

Post SPE Aliquot Vol. (mL) = 4.0

Post SPE Vol. (mL)	=	10
Final Vol. (mL)	=	2.0
DF	=	Dilution factor
1000 ng/µg	=	Conversion factor
Sample Weight (g)	=	2.5

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{\text{ppm found - ppm (> LOD) found in control}}{\text{ppm added}} \times 100$$

Note: Residue contribution of the control sample below 30% of the LOQ was not subtracted from the fortification residues.

Example: NF-171 recovery of grass clipping sample 81543-MV-096-B, Fortified Control @ 0.01 ppm, Set #MV8. See Appendix D.

The concentration determined from the standard curve is = 0.38167 ng/mL (as per Analyst 1.6.2)

The residue of NF-171 in the final solution is calculated as follows:

$$\text{NF-171 (ppm)} = \frac{0.38167\ \text{ng/mL} \times 60\ \text{mL} \times 10\ \text{mL} \times 2.0\ \text{mL} \times 1}{5.0\ \text{mL} \times 4.0\ \text{mL} \times 2.50\ \text{g} \times 1000\ \text{ng}/\mu\text{g}} = 0.00916\ \text{ppm}$$

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{\text{ppm found - ppm (> LOD) found in control}}{\text{ppm added}} \times 100$$

$$\text{Percentage Recovery} = \frac{0.00916\ \text{ppm} - 0.00\ \text{ppm}}{0.01\ \text{ppm}} \times 100 = 92\%\ \text{NF-171 recovery}$$

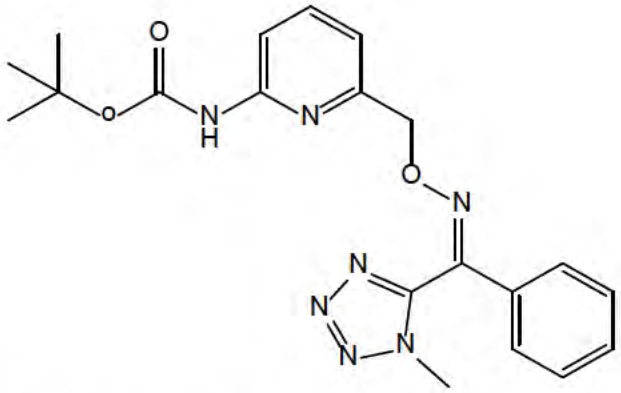
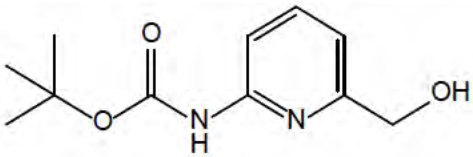
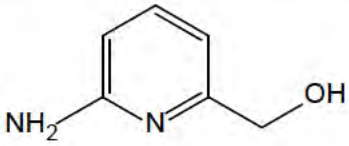
2.9 Confirmation of Residue Identity

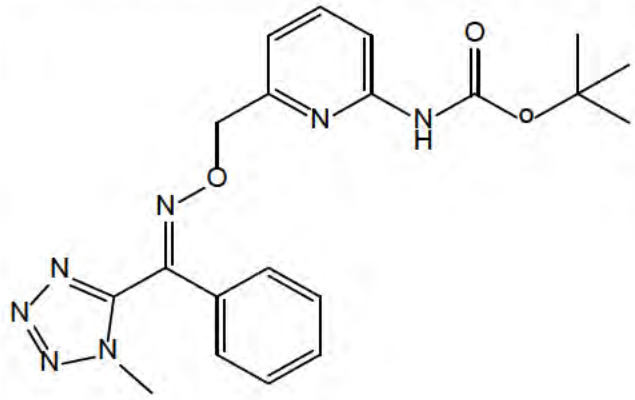
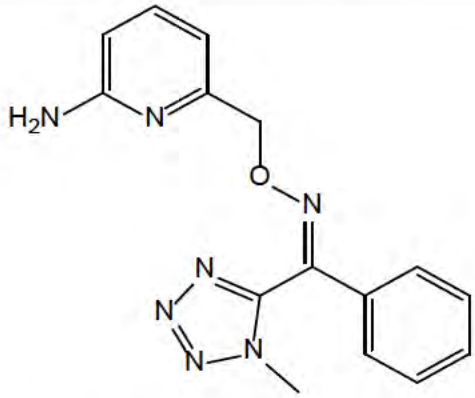
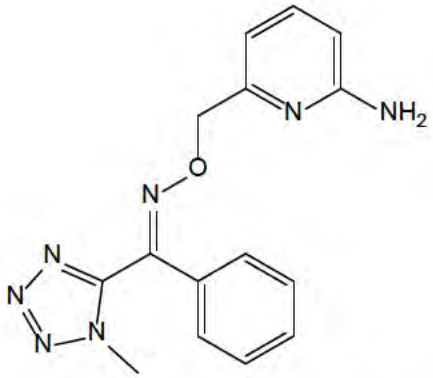
The method is specific for the determination of NF-171 and its metabolites by virtue of the chromatographic separation and selective detection system used (see Figure 1 through Figure 10). To demonstrate further confirmation, at least one additional MS/MS ion transition was monitored.

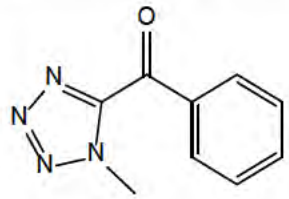
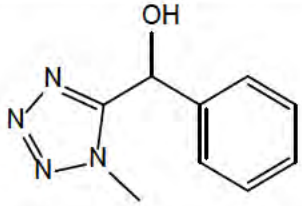
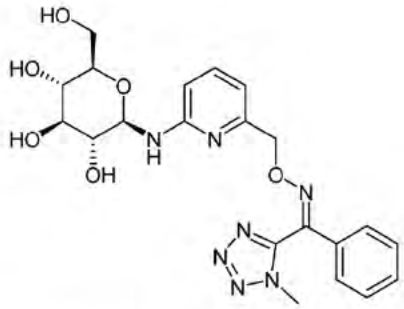
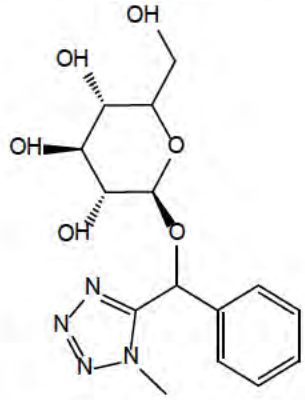
2.10 Statistical Treatment of Data

Statistical evaluations including percent recoveries, mean percent recoveries, and standard deviations were made using Microsoft Excel®.

Table 1 Identity and Structures of Picarbutrazox (NF-171) and Metabolites (TY-1, TY-2, TZ-1E, TZ-2, TZ-2E, TZ-4, TZ-5, TZ-2-β-Glc, and TZ-5-Glc)

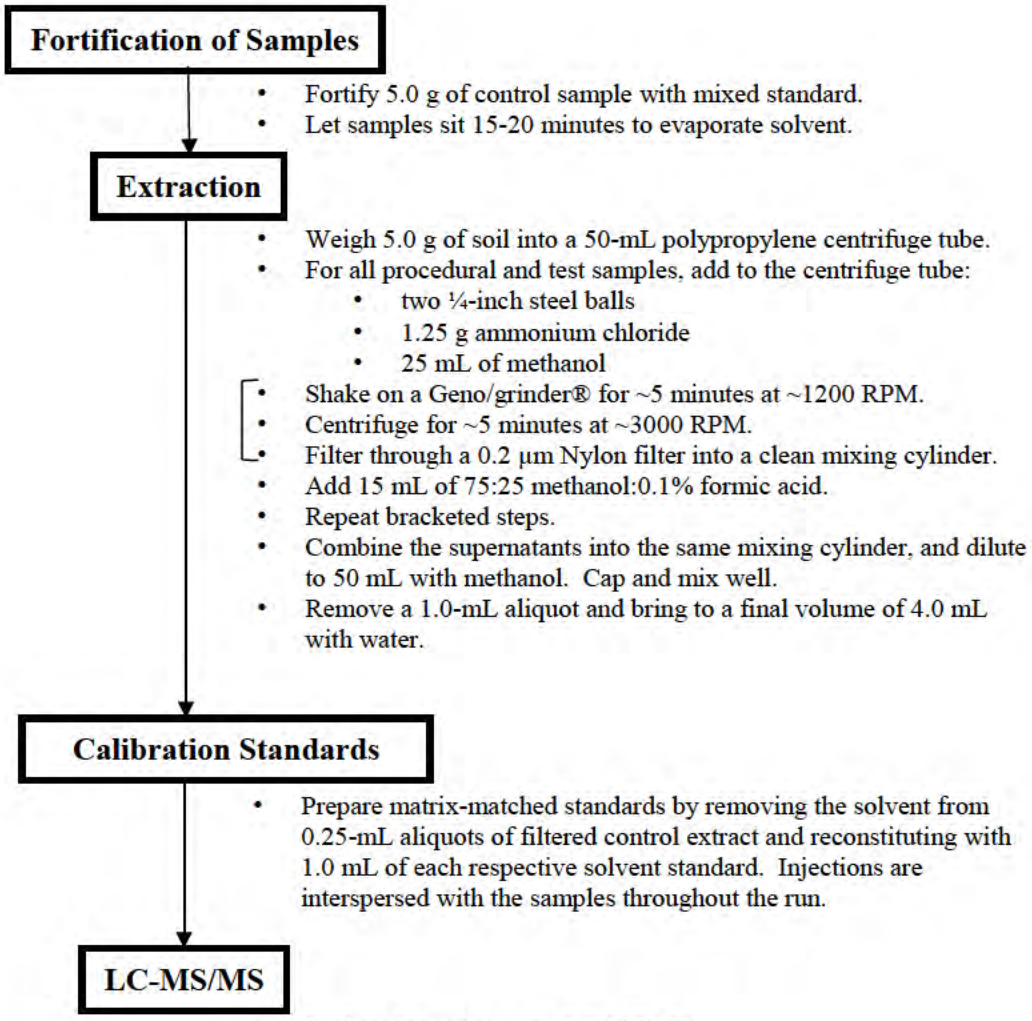
Common Name of Compound	Structural Formula and Chemical Name
Picarbutrazox (NF-171) Molecular Formula: $C_{20}H_{23}N_7O_3$ Formula Weight: 409.44 g/mol CAS Number 500207-04-5	 1,1-dimethylethyl *N*-[6-[[[(Z)-[(1-methyl-1*H*-tetrazol-5-yl)phenylmethylene]amino]oxy]methyl]-2-pyridinyl]carbamate
TY-1 Molecular Formula: $C_{11}H_{16}N_2O_3$ Formula Weight: 224.26 g/mol CAS Number 203321-83-9	 *tert*-butyl [6-(hydroxymethyl)pyridin-2-yl]carbamate
TY-2 Molecular Formula: $C_6H_8N_2O$ Formula Weight: 124.14 g/mol CAS Number 79651-64-2	 (6-amino-2-pyridyl)methanol

Common Name of Compound	Structural Formula and Chemical Name
TZ-1E Molecular Formula: $C_{20}H_{23}N_7O_3$ Formula Weight: 409.44 g/mol CAS Number 1253511-94-2	 *Tert*-butyl (6-{[(*E*)-(1-methyl-1*H*-5-tetrazolyl)(phenyl)methylene]-aminooxymethyl}-2-pyridyl)carbamate
TZ-2 Molecular Formula: $C_{15}H_{15}N_7O$ Formula Weight: 309.33 g/mol CAS Number 500206-79-1	 (*Z*)-*O*-[(6-amino-2-pyridyl)methyl](1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanone oxime
TZ-2E Molecular Formula: $C_{15}H_{15}N_7O$ Formula Weight: 309.33 g/mol CAS Number Not Registered	 (*E*)-*O*-[(6-amino-2-pyridyl)methyl](1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanone oxime

Common Name of Compound	Structural Formula and Chemical Name
TZ-4 Molecular Formula: $C_9H_8N_4O$ Formula Weight: 188.19 g/mol CAS Number 33452-25-4	 (1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanone
TZ-5 Molecular Formula: $C_9H_{10}N_4O$ Formula Weight: 190.20 g/mol CAS Number 33452-21-0	 (1-methyl-1*H*-5-tetrazolyl)(phenyl)-methanol
TZ-2-β-Glc Molecular Formula: $C_{21}H_{25}N_7O_6$ Formula Weight: 471.47 g/mol CAS Number Not Registered	 (*Z*)-*O*-{[6-(β-D-glucopyranosyl)amino-2-pyridyl]methyl]}(1-methyl-1*H*-5-tetrazolyl)(phenyl)methanone oxime
TZ-5-Glc Molecular Formula: $C_{15}H_{20}N_4O_6$ Formula Weight: 352.34 g/mol CAS Number Not Registered	 (1-methyl-1*H*-5-tetrazolyl)(phenyl)methyl-β-D-glucopyranoside

Appendix A: Method Flowcharts

SOIL ANALYSIS FLOWCHART



GRASS CLIPPINGS ANALYSIS FLOWCHART