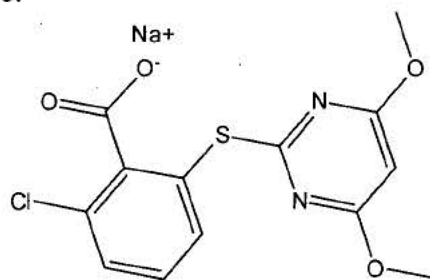
3.0 MATERIALS AND METHODS

3.1 Test Substances

The reference analytical standards (test substances) used for this study were:

Pyrithiobac Sodium:

DuPont Code: DPX-PE350-045
Chemical Name:
IUPAC: sodium 2-chloro-6-(4,6-dimethoxypyrimidin-2-ylthio)benzoate
CAS: sodium 2-chloro-6-[(4,6-dimethoxy-2-pyrimidinyl)thio]benzoate
CAS No.: 123343-16-8
Chemical Structure:

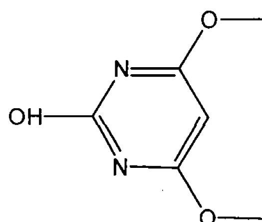


Pyrithiobac Sodium

Molecular Weight: 348.74 g/mole
Source: E. I. du Pont de Nemours and Company
Purity: 93.5% and 91.6%
Lot No.: E100076-124
Receipt Date: 19 June 2013
Expiration Dates: 21 July 2013 and 11 July 2016
Storage: Ambient

IN-B5363:

DuPont Code: IN-B5363-002
Chemical Name:
CAS: Not available
CAS No.: Not available
Chemical Structure:

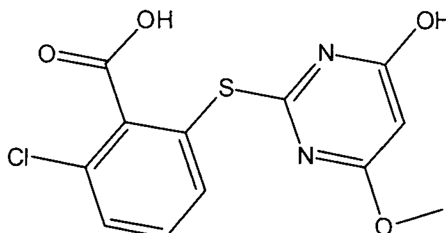


IN-B5363

Molecular Weight:	156.14 g/mole
Source:	E. I. du Pont de Nemours and Company
Purity:	97.5% (assumed 100%)
Lot No.:	E118883-36
Receipt Date:	23 August 2013
Expiration Date:	23 August 2015
Storage:	Ambient

IN-JW212:

DuPont Code: IN-JW212-002
Chemical Name:
CAS: Not available
CAS No.: Not available
Chemical Structure:



IN-JW212

Molecular Weight:	313.74 g/mole
Source:	E. I. du Pont de Nemours and Company
Purity:	94.9%
Lot No.:	2
Receipt Date:	19 June 2013
Expiration Date:	06 April 2016
Storage:	Ambient

Pyrithiobac sodium, IN-B5363 and IN-JW212 standards were supplied by E. I. du Pont de Nemours and Company, Newark, DE. Information pertaining to the characterization and stability of the test substances is archived by DuPont Crop Protection, E. I. du Pont de Nemours and Company, Newark, Delaware. The Certificates of Analysis are included in Appendix 2.

3.2 *Test Systems*

In this study, the analytical method was validated on soil, the matrix for which the method was designed.

Control soil samples used in the study were purchased from AGVISE Laboratories, Inc. in Northwood, ND. The samples were immediately placed into limited-access frozen storage at a temperature range of −20 ± 5°C. The samples remained in frozen storage until removed for subsampling and analysis. Samples were logged in according to ABC Laboratories' SOPs using the original sample numbers assigned to them. See Appendix 3 for the detailed soil characterization report.

3.3 *Equipment*

Equipment used is the same as that specified in the analytical method, except as follows:

Balances:	Mettler Model XP205DR, for weighing solid standards Mettler Model BB2440, for weighing soil samples
Centrifuge:	Beckman GP Benchtop Beckman Model GS 6R/HT
Genogrinder:	SPEX Sample Prep Model 2010
HPLC/MS System:	Applied BioSystems/MDS Sciex API 5000 LC/MS/MS with Waters Acquity system. The system is controlled and data processed by Applied BioSystems/MDS Sciex Analyst Software.
Pipets:	Gilson Microman, Gilson 1000 µL, Gilson 10-100 µL Positive Displacement, Hamilton 100 µL Air Displacement, Eppendorf 100-1000 µL Digital

3.4 *Reagents and Standards*

Reagents and standards used were of equivalent grade as that specified in the analytical method.

3.5 *Principles of the Analytical Method*

The residue analytical method described in DuPont-37904, entitled "Analytical Method for the Determination of Pyrithiobac Sodium and Metabolites in Soil Using LC/MS/MS," was used for the analyses in this study. See Appendix 1 for the complete text of the method as conducted at ABC Laboratories, Inc. The following is a summary of that method:

Pyrithiobac sodium and its metabolite residues were extracted from the soil sample three times by homogenization in, 1) 90:10 acetone:0.1M ammonium carbonate (aq), 2) 50:50 acetone:0.1M ammonium carbonate (aq), and 3) 20:80 acetone:0.1M ammonium carbonate (aq). The extract was brought to a final volume of 50 mL by adding 20:80 acetone:0.1M ammonium carbonate (aq). An aliquot was evaporated using an N-Evap with a water bath set to ~30°C until only the aqueous portion remained, then partitioned with 1:1 hexane:ethyl acetate. The upper layer was discarded before the extract was evaporated, diluted with water, and submitted for analysis by LC/MS/MS. Detection of the analytes was by electrospray mass spectrometry/mass spectrometry (ESI-MS/MS) in the positive ion mode.

3.6 Modifications, Interpretations, and Critical Steps

The method was performed with an LOQ of 0.0010 ppm, which is the intended LOQ of the method. It is recommended that Section 5.1.4 *Limit of Quantitation and Limit of Detection* be updated to reflect this LOQ and subsequent LOD.

Section 4.2.2 *Preparation of Solutions* gives a 1-month expiration date for all solutions. After some discussion with the Sponsor about the failure of IN-B5363 to meet acceptance criteria in Trial 1, it was determined that acetone:0.1 M aqueous ammonium carbonate solutions may need to be prepared on the same day extraction is performed. This procedure was implemented in Set #2. Since IN-B5363 passed with these preparations, this appears to be a critical step, and it is recommended the method be modified to reflect the possibility of poor performance with acetone:0.1 M aqueous ammonium carbonate solutions used beyond a 1 day expiration.

The analytical method was run exactly as written.

3.7 Instrumentation

The quantitative analysis of pyrithiobac sodium and its metabolites was performed using a Waters Acquity system coupled to an Applied BioSystems/MDS Sciex API 5000 LC/MS/MS system. The system parameters are shown in the tables below. Peak area was used for quantitation.

Typical HPLC Conditions:

System:	MDS Sciex API 5000 LC-MS/MS; Waters Acquity			
Column:	3.0 mm i.d. × 50 mm, ACE 3 C18-PFP analytical column with 3µm particle size			
Column Temperature:	40°C			
Injection Volume:	10 µL 3 µL (IN-B5363 only)			
Autosampler Temperature:	4°C			
Flow Rate:	0.60 mL/minute			
Mobile Phase:	A: 0.05% Formic acid in water B: Methanol			
Mobile Phase Conditions:	Time	%A	%B	Flow (mL/min)
	0.00	90	10	0.60
	4.00	90	10	0.60
	7.00	1	99	0.60
	9.00	1	99	0.60
	10.00	90	10	0.60
	17.00	90	10	0.60
Retention Times:	Pyrithiobac Sodium ~6.8 minutes IN-B5363 ~1.9 minutes IN-JW212 ~6.0 minutes			
Total Run Time:	~17.0 minutes			

A switching valve was not used for this method. The detection method utilized was LC-MS/MS employing electrospray (TIS) interface in the positive mode on a triple quadrupole instrument. The instrument was tuned by infusing the analytes into a TIS (turbo ion spray) source, then creating a tune file to maximize the response of each analyte using the TIS source. The acquisition method was adjusted to maximize the response of the fragment ions detected. The ion transitions for each analyte are shown in the table below:

MS Conditions:

System	Applied BioSystems/MDS Sciex API 4000 LC/MS/MS system						
Analytes Monitored	Ions Monitored (AMU)	Declustering Potential (volts)	Collision Energy (volts)	Dwell Time (seconds)	EP (volts)	CXP (volts)	Acquisition Timing (minutes)
Pyrithiobac Sodium	327.1 → 309.0[a]	80	23	150	10	20	6.8-7.1
	327.1 → 139.0[b]		42	150	10	20	
	327.1 → 83.0[b]		55	150	10	20	
IN-B5363	157.1 → 68.0[a]	85	33	150	10	10	1.8-2.1
	157.1 → 58.1[b]						
IN-JW212	313.1 → 196.0[a]	60	38	150	10	15	5.9-6.2
	313.1 → 295.0[b]		20	150	10	15	

[a]Transition ion used for quantitation
[b]Transition ion used for confirmation.

Additional detector settings are shown in the table below:

Parameter	**Setting**
Acquisition Mode:	MRM
Ionization Mode:	positive (+)
Source Temp.:	700°C
Nebulizer (GS1):	40
Auxiliary Gas (GS2):	50
Curtain Gas:	30
CAD Gas:	4
Ion Spray Voltage:	5500

The instrument was operated in the MS/MS (MRM) positive ion mode for quantitative analysis. Single transition chromatograms for each analyte were integrated and the peak areas used for quantitation. Quantitation was performed using a single transition for each analyte.

For each analytical run, a six-point standard curve was prepared by injecting constant volumes of standard solutions of a mixture of all three analytes. Constant volume injections were used for sample extracts as well. A curve check standard was typically injected every 3-4 sample injections.

3.8 Calculations

Calculations were performed as directed by the method. A validated software application was used to create a standard curve based on linear regression. Linear regression was monitored to support the response linearity of the mass spectrometer detector. The regression functions were used to calculate a best fit line (from a set of standard concentrations in ng/mL versus peak response) to demonstrate that a linear

relationship exists between analyte concentration and peak response, and that a response factor approach to calculation was appropriate.

The equation used for the least squares fit is:

$$y = mx + b$$

where:

y	=	peak response
x	=	ng/mL found for peak of interest
m	=	slope
b	=	y-intercept

Equations

The calculations for ppm found and percent recovery (for fortified samples) were:

1. The amount of analyte (in ppm) found in the sample was calculated according to the following equation:

$$ppm\ found = \frac{peak\ resp. \times Avg.\ Resp.\ Fact. \times mL\ FV \times \frac{mL\ solv.}{mL\ aliq.} \times HPLC\ dil.\ factor}{g\ samp.\ wt. \times 1000}$$

where:

peak resp.	=	peak area response of analyte in sample extract (corrected for control response, if applicable)
Avg. Resp. Fact.	=	average standard response factor of all the standards analyzed with the analytical set, where the standard response factor for each standard:

$$= \frac{standard\ concentration\ (ng/mL)}{Peak\ area\ response\ of\ standard}$$

mL FV	=	mL volume of final extract submitted to HPLC (4.0 mL)
mL solv.	=	volume of extraction solvent (50.0 mL)

mL aliq.	=	mL aliquot of initial extract processed through the procedure (10 mL)
g samp. wt.	=	grams of sample extracted (10 g)
1000	=	conversion factor for ng to µg
HPLC dil. factor	=	The magnitude of dilution required to bracket the response of the sample within the standard curve responses. No dilution = HPLC dilution factor of **1**

2. Percent recovery of fortified samples (procedural fortifications) was determined using the following equation:

$$\%\ Recovery = \frac{ppm\ found\ in\ fortified\ sample}{ppm\ added} \times 100$$

Example Calculations

Pyrithiobac sodium, IN-B5363, and IN-JW212, were calculated in exactly the same manner. Only examples of pyrithiobac sodium will be provided and thus serve to illustrate the calculations of all analytes in soil.

1. Sample RMN 0-6" + 0.001, Pyrithiobac Sodium, Set #1, 80148-003, Fortified Control @ 0.001 ppm:

$$sample\ peak\ response = 161764$$

$$Avg.\ Resp.\ Fact. = 0.00000230$$

$$ppm = \frac{161764 \times 0.00000230 \times 4.0\ mL \times \frac{50\ mL}{10\ mL} \times 1}{10\ g \times 1000}$$

$$ppm = 0.000743474$$

$$Reported\ ppm = 0.00074$$

$$\%\ Rec. = \frac{0.000743474\ ppm}{0.0010\ ppm} \times 100$$

$$= 74\%$$