3.0 MATERIALS AND METHODS

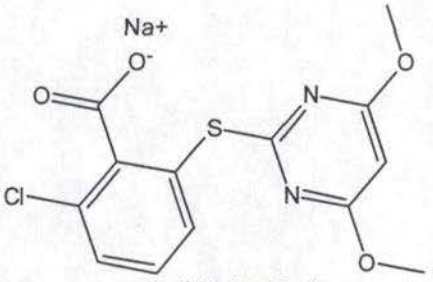
3.1 Test Substances

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:

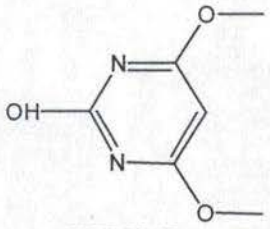


Pyrithiobac Sodium

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:



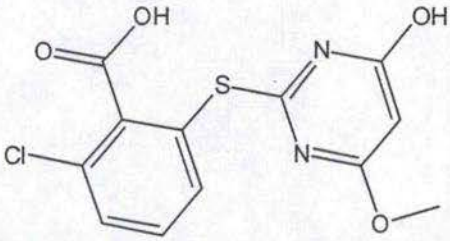
IN-B5363

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: 04 December 2016
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, DE. The Certificates of Analysis are included in Appendix 1.

3.2 *Test Systems*

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

Control water samples used in the study were purchased from AGVISE Laboratories, Inc. in Northwood, ND, except for the purified drinking water which was obtained from a local commercial source and was not characterized during the course of this study. The samples were immediately placed into limited-access refrigerated storage upon receipt, typically at 5°C. The samples remained in refrigerated storage until removed for subsampling and analysis. Samples were logged in according to ABC Laboratories' SOPs using the designation control surface, ground and purified drinking water. Additional designations such as "fortified control" were assigned as appropriate.

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 water samples
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, Gilson 50-250 µL, Gilson 3-25 µL, Positive Displacement, Hamilton 1000 µL Air Displacement

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-36965, entitled "Analytical Method for the Determination of Pyrithiobac and Metabolites in Water Using LC/MS/MS," (Reference 3) was used for the analyses in this study. The following is a summary of that method:

Pyrithiobac sodium and its metabolite residues were extracted from the water sample by filtration and submitted for analysis by LC/MS/MS. Detection of the analytes was by turbo ion spray mass spectrometry/mass spectrometry (TIS-MS/MS) in the positive ion mode.

3.6 Modifications, Interpretations, and Critical Steps

The analytical method was run exactly as written with the exception of a 6-port valve not being used as part of the LC-MS/MS system.

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.

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:	25 µL				
Autosampler Temperature:	5°C				
Flow Rate:	0.60 mL/minute				
Mobile Phase Conditions:	Time	%A	%B	Flow (mL/min)	A: 0.05% Formic acid in water B: Methanol
	0.00	90	10	0.60	
	2.00	90	10	0.60	
	5.00	1	99	0.60	
	7.00	1	99	0.60	
	8.00	90	10	0.60	
	15.00	90	10	0.60	
Retention Times:	Pyrithiobac Sodium			~4.93 minutes	
	IN-B5363			~2.37 minutes	
	IN-JW212			~4.14 minutes	
Total Run Time:	~15.0 minutes				

No switching was used for this method. The detection method utilized was LC-MS/MS employing turbo ion spray (TIS) interface in the positive mode on a triple quadrupole instrument. The acquisition method was adjusted to optimize 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 5000 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	326.9 → 309.0[a]	80	23	0.15	10	24	4.8-5.1
	329.0 → 139.1[b]		42	0.15	10	22	
IN-B5363	157.1 → 68.0[a]	85	33	0.15	10	10	2.1-3.2
	157.1 → 58.1[b]			0.15			
IN-JW212	313.1 → 196.0[a]	60	38	0.15	10	15	4.1-4.5
	313.1 → 295.0[b]		20	0.15	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. Quantitation and confirmatory transition chromatograms were integrated for each analyte, and the peak areas used for quantitation. Two ion transitions were monitored 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 ppb found and percent recovery (for fortified samples) were:

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

$$\text{ppb found} = \frac{\text{Peak Area} \times \text{RF Avg} \times \text{mL FV} \times \text{HPLC dil. factor}}{\text{Sample Weight}}$$

where:

Peak Area = peak area response of analyte in sample extract (corrected for control response, if applicable)

RF Avg = average standard response factor of all the standards analyzed with the analytical set, where the standard response factor for each standard:

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

mL FV = mL volume of final extract submitted to HPLC (10 mL)

Sample weight = grams of sample extracted (10 g)

HPLC dil. factor = 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:

$$\%\,\text{Recovery} = \frac{\text{ppb found in fortified sample}}{\text{ppb added}} \times 100$$

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 water.

1. Sample Control surface water + 0.1 ppb, Pyrithiobac Sodium, Set SW1, 80142-003, Fortified Control @ 0.10 ppb:

sample peak response = 175644

RF Avg = 0.000000540

$$\text{ppb} = \frac{175644 \times 0.000000540 \times 10\,\text{mL} \times 1}{10\,\text{g}}$$

$$\text{ppb} = 0.0948$$

$$\%\,\text{Rec.} = \frac{0.0948\,\text{ppb}}{0.10\,\text{ppb}} \times 100$$

$$= 95\%$$