

## 2 INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of pyridate and pyridafol in surface and ground water. The analysis of the pyridate and pyridafol reference/test substance was performed by liquid chromatography coupled with positive-ion tandem mass spectrometry (LC-MS/MS). The method validation report used to conduct this ILV was “Analytical Method Validation for the Determination of Pyridate and Pyridafol in Freshwater and Surface Water”, EAG Laboratories-Easton, Project No. 792C-105, November 1, 2018, provided by the sponsor.

This study was designed to satisfy US EPA Guideline requirements described in OCSP 850.6100. The study was initiated on November 2, 2018 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.

### 3 MATERIAL AND METHODS

#### 3.1 Test/Reference Substances

##### 3.1.1 Pyridate

IUPAC Name: *O*-6-chloro-3-phenylpyridazin-4-yl *S*-octyl thiocarbonate

CAS No: 55512-33-9

Molecular Formula: C<sub>19</sub>H<sub>23</sub>ClN<sub>2</sub>O<sub>2</sub>S

Molecular Weight: 378.9 g/mol

Lot Number: B18ZJ03131

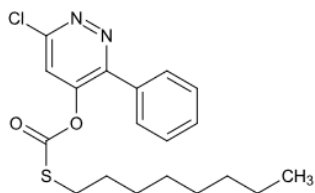
Purity: 99.5%

Expiration Date: March 12, 2021

Storage: cool, dry place (room temperature cabinet#7 at EAG-Hercules)

Inventory No.: 3113W-004

Structure:



##### 3.1.2 Pyridafol

IUPAC Name: 6-chloro-3-phenylpyridazin-4-ol

CAS No: 40020-01-7

Molecular Formula: C<sub>10</sub>H<sub>7</sub>ClN<sub>2</sub>O

Molecular Weight: 206.6 g/mol

Lot Number: B16ZJ11202

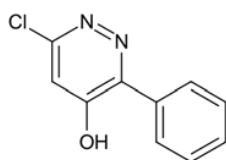
Purity: 99.9%

Expiration Date: November 19, 2018

Storage: 2-8°C (refrigerator #29 at EAG-Hercules)

Inventory No.: 3113W-003

Structure:



Pyridate and pyridafol standards were provided by EAG Laboratories – Easton on October 10, 2018. The certificates of analysis are provided in [Appendix B](#).

### **3.2 Solvents and Reagents**

All solvents used in this study were HPLC-grade or equivalent. All reagents were ACS reagent grade or higher quality.

Acetonitrile (ACN)

Formic Acid

Methanol

Water

### **3.3 Equipment/Materials List**

Laboratory Balances

Weighing funnels, plastic disposable

Vortex

Sonicator

Volumetric flasks and pipettes

Glass scintillation vials (20 mL capacity)

Plastic disposable centrifuge tubes (50 mL capacity)

Glass graduated disposable pipettes

Variable/adjustable volume pipettors with plastic disposable tips

Gas-tight precision syringes

0.45  $\mu\text{m}$  Rapid Flow Sterile filtration units with 0.45 $\mu\text{m}$  PES membrane (Thermo Scientific)

Amber glass bottles and vials (various sizes)

Autosampler vials

### **3.4 Instrumentation and HPLC Column**

Applied Biosystems MDS Sciex API4000 triple quadrupole LC-MS/MS system with Turbo Ionspray (ESI +) source

Thermo Scientific Dionex Ultimate 3000 (solvent degasser, binary HPLC pump, column oven and autosampler)

Analyst<sup>®</sup> Chromatography software (Sciex<sup>®</sup>) version 6.1.2.

Thermo BDS Hypersil C18 (50 mm, i.d.: 2.1 mm, particle size: 3µm)

### **3.5 Test Systems (Matrices) and Test System Preparation**

The surface water and groundwater (well water) were sourced by and obtained from EAG Laboratories – Easton. The groundwater and surface water test systems were received at EAG Laboratories – Hercules on August 3, 2018 and stored in a refrigerator (typically between 4 °C and 10°C) in the dark when not in use.

#### **3.5.1 Groundwater Source**

The groundwater was collected on August 7, 2018 in the aquatics lab at the Brooks Drive facility of EAG Laboratories – Easton (Easton, Maryland). The ground water was given the identification code of GRW-WL-080718. The ground water was characterized by Agvise Laboratories, Inc. (604 Highway 15 West, Northwood, North Dakota). The GLP characterization report is provided in [Appendix C](#).

#### **3.5.2 Surface Water Source**

The surface water was collected from the surface of Tuckahoe Lake located in Ridgely, Maryland on August 8, 2018. The surface water was given the identification code of SFW-TL-080718. The surface water was characterized by Agvise Laboratories. The GLP characterization report is provided in [Appendix C](#).

### **3.6 Determination of Pyridate and Pyridafol in Surface Water and Groundwater by LC-MS/MS**

#### **3.6.1 Preparation of Stock Solution**

Individual stock solutions of each test/reference substance were prepared by weighing the standards into separate disposable plastic weighing funnels on an analytical balance. The contents were dissolved in 100 mL volumetric flasks with appropriate solvent and sonication. The solutions were mixed well and transferred to amber glass jars for storage

in a freezer (typically between -10°C and -20°C) when not in use. Details of the stock solution preparation are provided in the following table:

EAG-Hercules Inventory no.	Standard Name	Weight (mg)	Final Volume (mL)	Purity (%)	Concentration (mg/mL) <sup>1</sup>	Solvent	Std ID
3113W-003A	Pyridafol	10.06	100	99.9	0.100499	Methanol	stock-1A
3113W-004	Pyridate	10.66	100	99.5	0.106067	0.1% formic acid in Methanol	stock-2A

<sup>1</sup> Concentration (mg/mL) = [weight (mg) ÷ final volume (mL)] x [purity (%) ÷ 100]

### 3.6.2 Preparation of Intermediate Solutions

Intermediate solutions were prepared by diluting the stock solutions with the appropriate solvent using volumetric pipets and 10-mL volumetric flasks. The solutions were vortex mixed and transferred to amber glass vials for storage in a freezer (typically between -10°C and -20°C) when not in use. Details of the intermediate solutions preparation are provided in the following table:

Solution Used	Standard Name	Aliquot (mL)	Final Volume (mL)	Concentration (mg/mL) <sup>2</sup>	Solvent	Std ID
stock-1A	Pyridafol	1.000	10.0	0.010050	Methanol	IM-1A
stock-1A		0.100	10.0	0.001005		IM-1B
stock-2A	Pyridate	0.950	10.0	0.010076	0.1% formic acid in Methanol	IM-2A
stock-2A		0.095	10.0	0.001008		IM-2B

<sup>2</sup> Concentration (mg/mL) = {[concentration of solution used (mg/mL) x aliquot (mL)] ÷ final volume (mL)}

### 3.6.3 Preparation of Calibration Standard Solutions

Six calibration solutions containing both pyridate and pyridafol were prepared in test system water. Surface water ILV samples were quantified using calibration solutions prepared using control surface water test system. Similarly, groundwater ILV samples were quantified using calibration solutions prepared using control groundwater test system. The calibration solutions were prepared by combining intermediate standard solutions of pyridate and pyridafol into volumetric flasks in the appropriate quantity using gas-tight syringes, then bringing the flasks to volume with acetonitrile/test system

water/0.1% formic acid (aq) (50/50/0.1, v/v/v). Calibrants were used on the day of preparation. Details of the calibrant preparation are provided in the following table:

Concentration of IM solns. (A or B) (mg/mL) <sup>3</sup>	Aliquot Intermediate Solution (µL)	Final Volume (mL)	Concentration <sup>4</sup> (ng/mL)	
			3113W-003A Pyridafol	3113W-004 Pyridate
0.001	25	25	1.01	1.01
0.001	125	25	5.03	5.04
0.001	250	25	10.1	10.1
0.01	50	25	20.1	20.2
0.01	90	25	36.2	36.3
0.01	125	25	50.3	50.4

<sup>3</sup> Approximate concentrations, for actual concentrations refer to intermediate solutions table.

<sup>4</sup> Concentration (ng/mL) = [IM solution conc. (mg/mL) x 1,000,000 ng/mg x [aliquot (µL) x 0.001 (mL/µL)] ÷ final volume (mL)

#### 3.6.4 Sample Preparation Procedure for Analysis

A flowchart of the sample preparation procedure is provided in [Figure 1](#).

1. Measure 10 mL matrix (surface water or groundwater) via 10 mL graduated cylinder into 20 mL glass scintillation vials. For a reagent blank use high purity water (HPLC grade water).
2. Fortify the samples as needed according to the following table:

Sample ID	Fortification Level (mg/L)	Concentration of Fort. Soln. Used (mg/L)	Fortification Volume (mL)
RB	NA	NA	NA
Control 1&2	NA	NA	NA
Fort 1A-> Fort 1E	0.005	1.0	0.050
Fort 2A-> Fort 2E	0.05	10.0	0.050

3. Immediately after fortification add 10 mL of 0.2% formic acid in acetonitrile. Mix well.
4. Aliquot a portion to autosampler vial for LC-MS/MS analysis.

Samples and associated calibrants were analyzed on same day of preparation.

### 3.6.5 LC-MS/MS Analysis Method

Acquisition method file “3113W-A1”

LC-MS/MS system:	Applied Biosystems MDS Sciex API4000 triple quadrupole LC-MS/MS system with Turbo IonSpray (ESI +) source, Thermo Scientific Dionex Ultimate 3000 (solvent degasser, binary HPLC pump, column oven and autosampler)																																											
HPLC column:	Thermo BDS Hypersil C18 (50 mm, i.d.: 2.1 mm, particle size: 3µm). Column temperature: 40°C.																																											
Injection volume:	5 µL																																											
HPLC Conditions:	Mobile phase A: 0.1% formic acid in HPLC grade H <sub>2</sub> O Mobile phase B: 0.1% formic acid in ACN Mobile Phase Composition: <table border="1" style="margin-left: 40px;"> <thead> <tr> <th>Time (min)</th> <th>Flow rate (µL/min)</th> <th>% A</th> <th colspan="2">% B</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>500</td> <td>85</td> <td colspan="2">15</td> </tr> <tr> <td>0.25</td> <td>500</td> <td>85</td> <td colspan="2">15</td> </tr> <tr> <td>2.0</td> <td>500</td> <td>40</td> <td colspan="2">60</td> </tr> <tr> <td>2.3</td> <td>500</td> <td>5</td> <td colspan="2">95</td> </tr> <tr> <td>5.5</td> <td>500</td> <td>5</td> <td colspan="2">95</td> </tr> <tr> <td>5.51</td> <td>500</td> <td>85</td> <td colspan="2">15</td> </tr> <tr> <td>9.0</td> <td>500</td> <td>85</td> <td colspan="2">15</td> </tr> </tbody> </table>				Time (min)	Flow rate (µL/min)	% A	% B		0	500	85	15		0.25	500	85	15		2.0	500	40	60		2.3	500	5	95		5.5	500	5	95		5.51	500	85	15		9.0	500	85	15	
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Retention time:	Pyridafol: 1.52 min Pyridate: 4.21 min																																											
Ion Source Conditions Turbo IonSpray (ESI) Positive Polarity	Source temperature: 550 °C Collision gas (CAD): 6 (arbitrary units) Curtain gas: 20 (arbitrary units) Nebulizer gas (GS1): 30 (arbitrary units) Turbo gas (GS2): 40 Declustering potential (DP): 36 V Focusing potential (FP): 160 V Entrance potential (EP): 10 V IonSpray voltage: 5500 V Interface heater: On																																											
Mass Spectrometer Conditions:	Resolution Q1: unit Resolution Q3: unit <table border="1" style="margin-left: 40px;"> <thead> <tr> <th>MS/MS transition</th> <th>Type</th> <th>Dwell Time (msec)</th> <th>CE</th> <th>CXP</th> </tr> </thead> <tbody> <tr> <td>Pyridate (379.1/207.1)</td> <td>Quantification</td> <td>150</td> <td>23</td> <td>10</td> </tr> <tr> <td>Pyridate (379.1/351.3)</td> <td>Confirmation</td> <td>150</td> <td>15</td> <td>10</td> </tr> <tr> <td>Pyridafol (207.0/104.1)</td> <td>Quantification</td> <td>150</td> <td>31</td> <td>4</td> </tr> <tr> <td>Pyridafol (207.0/126.1)</td> <td>Confirmation</td> <td>150</td> <td>49</td> <td>22</td> </tr> </tbody> </table>				MS/MS transition	Type	Dwell Time (msec)	CE	CXP	Pyridate (379.1/207.1)	Quantification	150	23	10	Pyridate (379.1/351.3)	Confirmation	150	15	10	Pyridafol (207.0/104.1)	Quantification	150	31	4	Pyridafol (207.0/126.1)	Confirmation	150	49	22															
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### 3.6.6 LC-MS/MS Analyses

For LC-MS/MS ILV surface water and groundwater sample analyses, calibrants were prepared in test system water and analyzed interspersed with ILV samples (either surface water or groundwater) from the lowest concentration to the highest concentration in single injections. A solvent blank and reagent blank was analyzed with each sequence. A single calibrant was re-analyzed at the end of the sequence as a quality control standard to ensure good chromatography and stable instrument signal. The QC standard was a repeat injection of one of the linearity calibrants (typically 10 ng/mL). The QC standard was acceptable if its calculated concentration accuracy (calculated by converting the peak area response to concentration using the linearity curve) is between 80% and 120%.

### 3.7 Methods of Calculation

Pyridate and pyridafol were quantitated by the external standard method using separate six-point linear curve regression for each compound and for each matrix. Separation and detection of the analytes was achieved by LC-MS/MS in MRM mode; Each compound was identified by the coincidence of retention time with their respective reference standard and MS characteristics. The quantitation of each compound was conducted by peak area relative to the theoretical concentration of the calibration standard solutions. The content of pyridate and pyridafol in surface and groundwater samples was quantified using separate  $1/x$  weighted linear curves ( $y = mx + b$ ) of pyridate and pyridafol calibration standards respectively where:

$y$  = peak area

$x$  = ng/mL compound injected

$m$  = slope

$b$  = intercept

Weighting of the calibration curves was applied so as to provide better curve fit at the lower concentration levels of the compounds. The calculation of weighted curve equations (linear regression) and concentration (ng/mL) present in samples and calibration standards was conducted using Analyst® software. The ng/mL final concentration determined from the calibration curve is then multiplied by a factor of 2 to account for the dilution factor during sample work up and dividing by a unit conversion factor of 1,000. Percent recovery is determined by comparing the calculated sample



concentration (mg/L) after subtracting average control concentration (if applicable) to the fortification concentration.

An example calculation of the analytical result and percent recovery for pyridate (primary transition) in pyridate fortified surface water validation (F1A, nominal concentration 0.005 mg/L) are presented below:

Final concentration of Pyridate in sample (mg/L) =

$$\frac{\text{peak area} - (\text{y-intercept})}{\text{slope}} \text{ (ng/mL)} \times \frac{\text{Final Volume (L)}}{\text{Water Sample Volume (L)}} \div 1,000 \text{ ng/}\mu\text{g}$$

Where  $\mu\text{g/mL} = \text{mg/L}$   
and

Peak area = 309000

y-intercept = -36200

Slope = 139000

Final Volume = 0.020 L

Water Sample Volume = 0.010 L

$$\text{Concentration of Pyridate (mg/L)} = \frac{309000 - (-36200)}{139000} \left(\frac{\text{ng}}{\text{mL}}\right) \times \left(\frac{0.020 \text{ L}}{0.010 \text{ L}}\right) \div 1,000 \text{ ng/}\mu\text{L}$$

Concentration of Pyridate (mg/L) in sample = 0.00497 mg/L

Percent of nominal concentration =

$$\frac{\text{measured concentration of sample} \left(\frac{\text{mg}}{\text{L}}\right) - \text{average measured concentration of matrix blank} \left(\frac{\text{mg}}{\text{L}}\right)}{\text{nominal concentration of sample} \left(\frac{\text{mg}}{\text{L}}\right)} \times 100$$

$$\text{Percent of nominal concentration} = \frac{0.00497 \frac{\text{mg}}{\text{L}} - 0.000540 \text{ mg/L}}{0.00500 \text{ mg/L}} \times 100$$

Where 0.000540 mg/L is average control pyridate residue

Percent of nominal concentration = 89%

\*Results were generated using Analyst Version 1.6.2. Manual calculations may differ slightly than those reported in the study file due to rounding and Analyst<sup>®</sup> hidden significant figures.

### **3.8 Time Required for Completion of a Sample Set**

A sample sets is defined as:

- 1 reagent blank sample
- 2 control matrix (matrix blank) samples
- 5 fortified control matrix samples @ LOQ
- 5 fortified control matrix samples @ 10xLOQ
- 6 matrix-matched calibrants

Time required for two sets from initiation of extraction until the completion of instrumental analysis and data evaluation for surface water and groundwater matrices is as follows:

- Preparation of stock and intermediate standard solutions takes approximately 2 hours for one analyst.
- Preparation of calibrants for each sample set takes one analyst approximately 0.75 hour.
- Preparation of two sets (calibrants and samples for LC-MS/MS analysis) takes one analyst approximately 7 hours.
- LC-MS/MS for 2 set takes approximately 3.5 hours (unattended).
- Data processing for LC-MS/MS takes one analyst approximately 1.5 hours

TOTAL = approximately 11 hours (1 calendar days) for one analyst to complete two sample sets to satisfy the validation requirements for surface or groundwater matrix.

### **3.9 Statistical Methods**

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

### **3.10 Communication Pertaining to Independent Laboratory Validation**

There was limited communication between the originating method validation laboratory and EAG-Hercules. The communication was coordinated through the Sponsor Representative and is summarized in [Appendix E](#).

### **3.11 Modifications of the Original Analytical Method**

No significant modification of the original analytical method was necessary. Minor modifications:

- The mass spectrometer source temperature setting was set at 550°C instead of 500°C.
- For each calibration curve, only one injection per calibrant concentration was made. The reference method indicates duplicate injections per calibrant as judged by the calibration curve figures ([Reference 1](#)).

Figure 1. Analytical Method Flowchart.

