

INTRODUCTION

Validation samples were fortified and analyzed to evaluate the accuracy and precision of a method for the analysis of pyridate and pyridafol in freshwater and surface water. The study was conducted by EAG Laboratories and identified as Project Number 792C-105. The study was performed based on procedures in *Residues: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, Section 4) and Annex III (Part A, Section 5) of Directive 91/414 (1)*, *Environmental Chemistry Methods and Associated Independent Laboratory Validation (2)*, and in the ICH Harmonised Tripartite Guideline, *Validation of Analytical Procedure: Text and Methodology Q2(R1) (3)*. The analysis of the samples was performed at EAG Laboratories using high performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS). Freshwater validation samples were prepared and analyzed between August 29 and September 19, 2018. Surface water validation samples were prepared and analyzed between September 6 and 7, 2018. Raw data generated by EAG Laboratories-Easton and the final report are filed under Project Number 792C-105 in archives located on the EAG Laboratories-Easton site.

PURPOSE

The purpose of this study was to validate the performance of methodology for analyses of pyridate and pyridafol in freshwater and surface water to be used by EAG Laboratories to perform environmental effects studies.

EXPERIMENTAL DESIGN

Freshwater and surface water samples were fortified at two different concentrations with pyridate or pyridafol and analyzed based on a method developed by EAG Laboratories. Two reagent blanks and two matrix blanks were analyzed concurrently to evaluate potential analytical interferences. Quantitation was performed with external standards of the test substance using concentrations bracketing the final concentrations of samples at the instrument. Calibration curves were prepared from external standards of pyridate and pyridafol to determine the test substance concentrations in the samples.

MATERIALS AND METHODS

This study was conducted according to the protocol “Analytical Method Validation for the Determination of Pyridate and Pyridafol in Freshwater and Surface Water” ([Appendix 1](#)).

Test Substances

The first test substance was received from BOC Sciences on April 27, 2018. It was assigned EAG Laboratories identification number 14684 upon receipt and was stored under ambient conditions. The test substance, a solid, was identified as: Pyridate; Batch No.: B18ZJ03131 and had a purity of 99.5%. The test substance had a retest date of March 12, 2021. The Certificate of Analysis is presented in [Appendix 2](#).

The second test substance was received from BOC Sciences on May 15, 2018. It was assigned EAG Laboratories identification number 14758 upon receipt and was stored refrigerated. The test substance, a solid, was identified as: 6-Chloro-4-hydroxy-3-phenyl-pyridazine; Batch No.: B16ZJ11202 and had a purity of 99.9%. The test substance had a retest date of November 19, 2018. The Certificate of Analysis is presented in [Appendix 2](#).

Reagents and Solvents

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

Test Systems

Freshwater

The water used for testing was freshwater obtained from a well approximately 40 meters deep located on the EAG Laboratories site in Easton, Maryland. The well water was passed through a sand filter to remove particles greater than approximately 25 μm , and pumped into a 37,800-L storage tank where the water was aerated with spray nozzles. Prior to use in the method verification, the water was filtered to 0.45 μm to remove fine particles and was passed through an ultraviolet (UV) sterilizer. The well water is characterized as moderately-hard water. The results of periodic analyses performed to measure the concentrations of selected organic and inorganic constituents in the well water are presented in [Appendix 4](#).

Surface Water

The surface water used for testing was freshwater obtained from the surface of Tuckahoe Lake, located in Ridgely, MD. This was collected in a 5 gallon carboy on August 7, 2018 and was given the identification code of SFW-TL-080718 and was assigned an expiration date of August 7, 2020. Approximately 1000 mL of the water was sent to Agvise Labs for GLP characterization ([Appendix 3](#)). The surface water was stored under refrigerated conditions when not in use.

Analytical Method

The methods used for the analysis of pyridate and pyridafol in freshwater and surface water were developed by EAG Laboratories. The analytical method for the freshwater analyses consisted of fortifying samples with the appropriate stock solutions. Following fortification, 0.2% formic acid in acetonitrile was added to each sample. An aliquot of each sample extract was transferred to autosampler vials and submitted for analysis by LC/MS/MS. The aliquots for samples fortified with pyridate were analyzed for pyridate and pyridafol. The aliquots for samples fortified with pyridafol were analyzed for pyridate and pyridafol.

The analytical method for the surface water analyses consisted of fortifying samples with the appropriate stock solutions. Following fortification, 0.2% formic acid in acetonitrile was added to each sample. An aliquot of each sample extract was transferred to autosampler vials and submitted for analysis by LC/MS/MS. The aliquots for samples fortified with pyridate were analyzed for pyridate and pyridafol. The aliquots for samples fortified with pyridafol were analyzed for pyridate and pyridafol.

Concentrations of pyridate and pyridafol in the samples were measured using a Sciex API 4000 LC/MS/MS coupled with an Agilent 1200 Series Infinity HPLC System. Chromatographic separations were achieved using a Thermo Betasil C-18 analytical column (50 mm x 2.1 mm, 3 μ m particle size) and a Thermo Betasil C-18 guard column (10 x 2.1 mm). The method flowchart for the analysis of pyridate and pyridafol in freshwater and surface water is presented in [Figure 1](#), and typical instrumental parameters are summarized in [Table 1](#).

Stocks/Standards Preparation

A stock solution of pyridate (test substance number 14684) was prepared by accurately weighing 0.0100 g of the test substance on an analytical balance. The test substance was transferred to a 100 mL volumetric flask and the contents were brought to volume using 0.1% formic acid in methanol, then sonicated for approximately one minute. The primary stock solution (0.100 mg/mL) (stock id: 14684-004; expiration date: March 1, 2019) was diluted in 0.1% formic acid in methanol to prepare 0.0100 (14684-004A; expiration date: March 1, 2019) and 0.00100 (14684-004B; expiration date: March 1, 2019) mg/mL stock solutions. The 0.0100 (14684-004A) and 0.00100 (14684-004B) mg/mL stock solutions were used to prepare the pyridate method verification samples and combined pyridate and pyridafol calibration standards for the analyses in freshwater and surface water.

A stock solution of pyridafol (test substance number 14758) was prepared by accurately weighing 0.0050 g of the test substance on an analytical balance. The test substance was transferred to a 50.0 mL volumetric flask and the contents were brought to volume using methanol, then sonicated for approximately five minutes. The primary stock solution (0.10 mg/mL) (stock id: 14758-003; expiration date: November 19, 2018) was diluted in methanol to prepare 0.010 (14758-003A; expiration date: November 19, 2018) and 0.0010 (14758-003B; expiration date: November 19, 2018) mg/mL stock solutions. A second stock solution of pyridafol was prepared by accurately weighing 0.0500 g of the test substance on an analytical balance. The test substance was transferred to a 50.0 mL volumetric flask and the contents were brought to volume using methanol, then sonicated for approximately five minutes. The primary stock solution (1.00 mg/mL) (stock id: 14758-004; expiration date: November 19, 2018) was diluted in methanol to prepare 0.0100 (14758-004A; expiration date: November 19, 2018) and 0.00100 (14758-004B; expiration date: November 19, 2018) mg/mL stock solutions. The 0.010 (14758-003A), 0.0100 (14758-004A), 0.0010 (14758-003B), and 0.00100 (14758-004B) mg/mL stock solutions were used to prepare the combined pyridate and pyridafol calibration standards. The 0.0100 (14758-004A) and 0.00100 (14758-004B) mg/mL stock solutions were used to prepare the method verification samples for the pyridafol analyses in freshwater and surface water.

The 0.00100 mg/mL stock solution of pyridate (14684-004B) and 0.0010 mg/mL pyridafol (14758-003B) and the 0.0100 mg/mL stock solutions of pyridate (14684-004A) and 0.010 mg/mL pyridafol (14758-003A) were used to prepare the combined calibration standards. Combined calibration standards were prepared in 50 : 50 : 0.1 (v/v/v) acetonitrile : freshwater : formic acid. The following shows the dilution scheme for the combined calibration standards for pyridate analysis in freshwater (monitoring pyridafol):

<u>Analyte</u>	<u>Stock Concentration (mg/mL)</u>	<u>Aliquot (μL)</u>	<u>Final Volume (mL)</u>	<u>Standard Concentration (mg/L)</u>
Pyridate	0.00100	50.0	50.0	0.00100
Pyridafol	0.0010	50.0		0.0010
Pyridate	0.00100	250	50.0	0.00500
Pyridafol	0.0010	250		0.0050
Pyridate	0.00100	500	50.0	0.0100
Pyridafol	0.0010	500		0.010
Pyridate	0.0100	100	50.0	0.0200
Pyridafol	0.010	100		0.020
Pyridate	0.0100	175	50.0	0.0350
Pyridafol	0.010	175		0.035
Pyridate	0.0100	250	50.0	0.0500
Pyridafol	0.010	250		0.050

The 0.00100 mg/mL stock solutions of pyridate (14684-004B) and pyridafol (14758-004B) and the 0.0100 mg/mL stock solutions of pyridate (14758-004A) and pyridafol (14758-004A) were used to prepare the combined calibration standards. Combined calibration standards were prepared in 50 : 50 : 0.1 (v/v/v) acetonitrile : freshwater : formic acid, or 50 : 50 : 0.1 (v/v/v) acetonitrile : surface water : formic acid appropriately. The following shows the dilution scheme for the combined calibration standards for the pyridafol analysis in freshwater (monitoring pyridate), pyridate analysis in surface water (monitoring pyridafol) and pyridafol analysis in surface water (monitoring pyridate):

<u>Analyte</u>	<u>Stock Concentration (mg/mL)</u>	<u>Aliquot (µL)</u>	<u>Final Volume (mL)</u>	<u>Standard Concentration (mg/L)</u>
Pyridafol	0.00100	50.0	50.0	0.00100
Pyridate	0.00100	50.0		0.00100
Pyridafol	0.00100	250	50.0	0.00500
Pyridate	0.00100	250		0.00500
Pyridafol	0.00100	500	50.0	0.0100
Pyridate	0.00100	500		0.0100
Pyridafol	0.0100	100	50.0	0.0200
Pyridate	0.0100	100		0.0200
Pyridafol	0.0100	175	50.0	0.0350
Pyridate	0.0100	175		0.0350
Pyridafol	0.0100	250	50.0	0.0500
Pyridate	0.0100	250		0.0500