INTRODUCTION

EAG Laboratories-Easton performed an independent laboratory validation (ILV) of a method for the determination of residues of Ipconazole in saltwater with adaption/extension of the method to include additional validations in surface, ground, and drinking water aqueous matrices. The protocol for this study titled "Independent Laboratory Validation of a Method for the Determination of Ipconazole in Aqueous matrices by LC-MS/MS" is presented in Appendix I. The final report of the developing lab EAG Laboratories-Columbia analytical method report entitled, "Method Validation for the Analysis of Ipconazole in Salt Water" is presented in Appendix II.

This study was performed to satisfy regulatory requirements for independent laboratory validation of methods as set forth by the U.S. Environmental Protection Agency Series 860 - Residue Chemistry Test Guidelines, OCSPP 850.6100, *Environmental Chemistry Methods and Associated Independent Laboratory Validation* (1) and U.S. Environmental Protection Agency, 1996. Pesticide Regulation (PR) Notice 96-1: Notice to Manufacturers, Formulators, Producers and Registrants of Pesticides Products, *Tolerance Enforcement Methods - Independent Laboratory Validation By Petitioner* (2). The study was performed at the EAG Laboratories analytical chemistry facility in Easton, Maryland. The experimental portion of the study was conducted between August 27, 2018 and October 16, 2018. Raw data and a copy of the final report are archived at the EAG Laboratories-Easton site under project number 556K-101.

PURPOSE

This study was conducted to fulfill EPA requirements set forth in guideline OCSPP 850.6100 and PR Notice 96-1. This study provides validation data demonstrating that an independent researcher could reproduce the results of the analytical method with minimal contact with the method developers.

EXPERIMENTAL DESIGN

Saltwater, surface water, ground water and drinking water matrices were fortified with Ipconazole test substance at two concentrations and analyzed according to a method supplied by the Sponsor. The limit of quantitation (LOQ) for Ipconazole was set at 0.0500 µg/L. The higher concentration was ten-fold the LOQ, i.e., 0.500 µg/L, respectively. Reagent and matrix blanks (controls) were prepared and analyzed concurrently with the fortified samples to evaluate potential analytical interferences.

MATERIALS AND METHODS

Untreated Control Matrices Origin - Salt water, Surface Water, Ground Water, and Drinking Water

Saltwater control matrix used for this study was natural seawater collected by EAG Laboratories-Easton from the Indian River Inlet located in Delaware. The water was filtered and diluted to a salinity of approximately 20‰ with well water prior to use. The saltwater was collected on September 07, 2018 from the aquatics laboratory designated spigot and was assigned the EAG laboratories identification code of SLW-IR-090718. The saltwater was logged into the EAG Laboratories-Easton glebe testing facility and stored under refrigerated conditions when not in use. The saltwater was characterized externally by Agvise Labs and a summary report is presented in Appendix III.

Surface water control matrix used for this study was obtained locally by EAG Laboratories-Easton from Tuckahoe Lake located in Tuckahoe State Park in Ridgely, MD. The surface water was collected on August 07, 2018 and was assigned the EAG Laboratories identification code of SFW-TL-080718. The surface water was logged into the EAG Laboratories-Easton glebe testing facility and stored under refrigerated conditions in the dark when not in use. The surface water was characterized externally by Agvise Labs and a summary report is presented in Appendix IV.

Ground water control matrix used for this study was obtained locally from a well at EAG Laboratories-Easton testing facility in Easton, MD. The ground water was collected on August 07, 2018 and was assigned the EAG Laboratories identification code of GRW-WL-080718. The ground water was logged into the EAG Laboratories-Easton glebe testing facility and stored under refrigerated conditions in the dark when not in use. The ground water was characterized externally by Agvise Labs and a summary report is presented in Appendix V.

Drinking water control matrix used for this study was obtained locally from a tap at EAG Laboratories-Easton glebe testing facility in Easton, MD. The drinking water was collected on August 07, 2018 and was assigned the EAG Laboratories identification code of DRW-TP-080718. The drinking water was logged into the EAG Laboratories-Easton glebe testing facility and stored under refrigerated conditions in the dark when not in use. The drinking water was characterized externally by Agvise Labs and a summary report is presented in Appendix VI.

Analytical Test/Reference Substances

A test substance of Ipconazole TG was received from Kureha Corporation on May 17, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13817. The material was a solid and was identified on the label and certificate of analysis as Ipconazole TG; Lot# 89010; Purity 96.7% (Ipconazole cc 89.7%, Ipconazole ct 7.0%); CAS Number 125225-28-7; Expiration Date November 24, 2019. This test substance was stored under ambient conditions. A certificate of analysis is presented in Appendix VII.

A reference substance of Ipconazole cc isomer was received from Kureha Corporation on May 17, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13819. The material was a solid and was identified on the label and certificate of analysis as Ipconazole cc; Lot# G-00328; Purity 99.5%; Expiration Date September 12, 2019. This test substance was stored under ambient conditions. A certificate of analysis is presented in Appendix VIII.

A reference substance of Ipconazole ct isomer was received from Kureha Corporation on May 17, 2017 and was assigned the EAG Laboratories-Easton Identification Number 13820. The material was a solid and was identified on the label and certificate of analysis as Ipconazole ct; Lot# G-00329; Purity 99.7%; Expiration Date September 09, 2021. This test substance was stored under ambient conditions. A certificate of analysis is presented in Appendix IX.

The test substance was used to a prepare primary analytical stock and subsequently various secondary fortification stock solutions of Ipconazole TG for preparation of method validation samples and the reference substances were used to prepare separate primary analytical stocks and subsequently various secondary calibration stocks and standard solutions of Ipconazole cc and ct isomers for quantitation of the test substance as discussed below.

Preparation of Primary Test Substance and Analytical Reference Stocks, Secondary Fortification and Calibration Stocks, and Working Calibration Standards

A primary stock solution of Ipconazole TG test substance was prepared by weighing a 0.1034 g aliquot onto a piece of tared weigh paper. The test material was transferred to a 25-mL volumetric flask, dissolved and adjusted to final volume using acetonitrile to yield a final nominal stock concentration of 4.00 mg/mL (corrected for purity).

Secondary fortification stocks of Ipconazole TG test substance for fortification of validations samples were prepared at 40.0, 0.100 and 0.0200 µg/mL in acetonitrile solvent as shown below:

Stock Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Fortification Stock Concentration (µg/mL)
4000	1.00	100	40.0
40.0	0.250	100	0.100
40.0	0.0500	100	0.0200

A primary stock solution of Ipconazole cc isomer reference substance was prepared by weighing a 0.01005 g aliquot onto a piece of tared weigh paper. The test material was transferred to a 25-mL volumetric flask, dissolved and adjusted to final volume using acetonitrile to yield a final nominal stock concentration of 0.400 mg/mL (corrected for purity).

A secondary calibration stock of Ipconazole cc isomer reference substance for the preparation of working calibration standards was prepared in methanol: HPLC grade water (50:50, v/v) as shown below:

Stock Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Calibration Stock Concentration (ng/mL)
400	0.0780	25.0	1250

Working calibration standards of Ipconazole cc isomer ranging in concentration from 0.0500 to 10.0 ng/mL were prepared in in methanol: HPLC grade water (50:50, v/v) from the secondary calibration stock above as shown below:

Secondary Stock Concentration (ng/mL)	Aliquot (mL)	Final Volume (mL)	Working Calibration STD Conc. (ng/mL)
1250	0.200	25.0	10.0*
1250	0.100	25.0	5.00
1250	0.0500	25.0	2.50
10.0*	2.50	25.0	1.00
10.0*	1.25	25.0	0.500
10.0*	0.500	25.0	0.200
10.0*	0.250	25.0	0.100
10.0*	0.125	25.0	0.0500

*Note: 10.0 ng/mL working calibration standard level was used to prepare the lower level calibration standards as shown.

A primary stock solution of Ipconazole ct isomer reference substance was prepared by weighing a 0.01003 g aliquot onto a piece of tared weigh paper. The test material was transferred to a 25-mL volumetric flask, dissolved and adjusted to final volume using acetonitrile to yield a final nominal stock concentration of 0.400 mg/mL (corrected for purity).

A secondary calibration stock of Ipconazole cc isomer reference substance for the preparation of working calibration standards were prepared at in methanol: HPLC grade water (50:50, v/v) as shown below:

Stock Conc. (µg/mL)	Aliquot (mL)	Final Volume (mL)	Calibration Stock Concentration (ng/mL)
400	0.125	50.0	1000

Working calibration standards of Ipconazole ct isomer ranging in concentration from 0.0100 to 5.00 ng/mL were prepared in in methanol: HPLC grade water (50:50, v/v) from the secondary calibration stock as shown below:

Secondary Stock Concentration (ng/mL)	Aliquot (mL)	Final Volume (mL)	Working Calibration STD Conc. (ng/mL)
1000	0.125	25.0	5.00*
5.00*	5.00	25.0	1.00
5.00*	2.50	25.0	0.500
5.00*	1.00	25.0	0.200
5.00*	0.500	25.0	0.100
5.00*	0.250	25.0	0.0500
5.00*	0.100	25.0	0.0200
5.00*	0.0500	25.0	0.0100

*Note: 5.00 ng/mL working calibration standard level was used to prepare the lower level calibration standards as shown.

All solutions were prepared using volumetric flasks, gas-tight syringes, and/or assorted sized digital pipettors. All stock/standard solutions were transferred to amber glass vials or bottles and stored under refrigerated conditions when not in use.

Analytical Method – Aqueous Matrices

The analytical method for the extraction and analysis of Ipconazole in aqueous matrices employed a solid phase extraction (SPE) procedure to extract, clean-up, and concentrate the samples. Final quantitation of samples was performed utilizing High Performance Liquid Chromatography with tandem mass spectrometric detection (HPLC/MS/MS).

Fortification of Recovery Samples

For each of the aqueous matrix validations, one reagent blank, two unfortified matrix blanks, five fortified control matrix samples at the LOQ, and five fortified control matrix samples at 10X the LOQ were prepared in ground and surface water as shown below:

Ipconazole Fortification Table

Nominal Concentration (µg/L)	Fortification Volume (mL)	Sample Volume (mL)	Fortification Stock Conc. (µg/mL)
0.0500 (LOQ)	0.0500	20.0	0.0200
0.500 (10X LOQ)	0.100	20.0	0.100

All fortified samples and calibration standards were prepared with stock solutions that were prepared compensating for the purity of the test/reference materials. Therefore, residue fortification and recovery levels, expressed in µg/L, are equivalent to the expression as µg/L active ingredient (µg/L a.i.).

Extraction and Analysis of Ipconazole from Aqueous Matrices

For analysis, 20.0-mL volumes of appropriate aqueous control matrix were measured into thirteen individually labeled 20-mL culture tubes, five of which were fortified with Ipconazole TG at the LOQ (0.0500 µg/L) and five at 10x the LOQ (0.500 µg/L) with secondary fortification stocks of the test substance prepared as described above. A single reagent blank consisting HPLC grade reagent water and the two matrix blanks of unfortified control matrix were also prepared and carried through the methodology for each matrix. One milliliter of acetonitrile solvent was added to each sample, followed by mixing. All samples were subsequently analyzed by methodology in Appendix II. Slight deviations in the LC/MS/MS source optimization parameters and chromatographic conditions were utilized and were considered to be equivalent values related to inherent differences in instrumental performance and not a limitation of the methodology. Since specific details of the method are presented in Appendix II, a more general description is provided here.

For the SPE extraction/clean-up, an appropriate number of Waters Sep-pak C_{18} Vac SPE cartridges (6cc, 1.0g) were conditioned with 5 mL of acetonitrile, followed by 5 mL of HPLC grade water, discarding the eluate to waste (Cartridges were not allow to go dry). The prepared validation samples above were loaded onto each conditioned SPE cartridge by gravity at a steady rate, discarding the eluates. (Note: SPE flow rate is critical to the success of this analysis, both during loading and elution steps. Flow rate should be minimized to less than or equal to 1 drop/second). Next, 5.0 mL volumes of HPLC grade water was added to each sample container as a rinse and then added as a wash to each SPE cartridge. The eluates were discarded and air was drawn drawn through the SPE cartridges using vacuum for a few minutes to remove any residual water. The analyte was eluted from the cartridges by gravity using 8.0 mL of acetonitrile, collecting the eluates into a 10-mL culture tube or equivalent. Using vacuum, air was again drawn through the SPE cartridges for a few minutes to remove any residual acetonitrile elution solvent and 20 µL volumes of octan-1-ol were added to each tube. The extracts were evaporated to near dryness under a steady stream of nitrogen using a nitrogen evaporator set at a bath temperature of approximately 45° C. The sample residues were reconstituted initially using 1.00 mL volumes of methanol with the aid of vortex mixing and ultra-sonication and then diluted further to a 2.00 mL final volume using HPLC grade water and vortex mixing. The final reconstituted sample extracts were transferred to auto-sampler vials and submitted for HPLC-MS/MS analysis.

Quantitation of Ipconazole by HPLC/MS/MS

An Agilent Technologies Model 1200 Infinity Series High Performance Liquid Chromatograph connected to an AB Sciex API 5000 Mass Spectrometric Detector (LC/MS/MS) was used to analyze samples. A

0.1mM formic acid + 0.1mM in HPLC grade water: methanol gradient was used. The Ipconazole test substance chromatographed as two primary, separated isomers (Ipconazole cc and Ipconazole ct). The measured concentrations of each individual isomer were calculated using separate reference substances of each and the results were summed to quantitate the residue results as total Ipconazole TG test substance.

Quantitation was performed using the response of the primary ion transitions for Ipconazole cc and Ipconazole ct isomers. Confirmation analysis was performed using the response of the secondary confirmation ion transition for each isomer as well. The ion transitions monitored are summarized below:

Analyte	Primary Transition (Quantitation)	Secondary Transition (Confirmation)
Ipconazole cc	334.1→70.1 amu	334.1→125 amu
Ipconazole ct	334.1→70.1 amu	334.1→125 amu

Specific details of the HPLC/MS/MS instrumentation and operational parameters are presented in Table 1.

Method Detection Limit (MDL), Practical Quantitation Limit (PQL), Minimum Quantitation Limit (MQL), and Limit of Quantitation (LOQ)

The Method Detection Limit (MDL) and Practical Quantitation Limit (PQL) were determined for Ipconazole cc and Ipconazole ct isomers (both quantitation and confirmation ion analyses) by analysis of seven replicate injections of the lowest calibration standard for each isomer. The MDL was calculated as 3.143 times the standard deviation of the seven replicate injections, expressed as µg/L. The PQL, the lowest detection limit that is routinely achievable among laboratories during routine operation, was calculated as 5 times the MDL value. The MQL for each Ipconazole isomer was calculated as the product of the concentration of the lowest calibration standard times the dilution factor of the control samples. The LOQ was set at 0.0500 µg/L Ipconazole TG, the lowest level fortified and validated during the method validation study.

Matrix Effects Evaluation

The evaluation of salt water matrix effects was determined in the original ECM method validation and were shown to be benign based on the lack of control residues and acceptable fortified recoveries achieved (<20 enhancement or suppression). For the additional method adaptation/extension matrices of surface, ground, and drinking water, the matrix effects were evaluated by comparison of replicate injections of a solvent based calibration standard verses a LOQ matrix-matched calibration standard in each of the three matrices.

Example Calculations

Calculation of Ipconazole TG total test substance concentrations were derived from separate Ipconazole cc and Ipconazole ct analysis in the method validation samples. For each Ipconazole isomer analyte (cc/ct), a separate regression equation was derived from the chromatographic peak area responses of each analyte determined in calibration standard solutions versus the respective nominal concentrations of the standards. Standard curves were generated by plotting this function with analyte concentration (µg/L) on

the abscissa and the respective analyte peak area response on the ordinate. The applied regression was weighted 1/x with respect to concentration and expressed as a linear regression as follows:

$$y = mx + b$$

Where:
- Y = peak area
- m = slope
- b = Y-intercept
- x = analyte concentration

Concentrations of Ipconazole cc and Ipconazole ct isomers in the samples (quantitation and confirmation analyses) were determined separately by substituting peak area responses of the samples into the applicable re-arranged weighted (1/x) regression equation as follows:

$$\text{Analyte Concentration} = \frac{\text{Peak area - (Y-intercept)}}{\text{Slope}}$$

Using the data from the salt water method validation sample 556K-101-SFW-VMAS-11, 0.0500 µg/L shown below, the analytical result and percent recovery was calculated as follows using the software algorithms of Analyst version 1.6 of the AB Sciex API 5000 mass spectrometer system in full precision mode. Note: manual calculations shown here may differ slightly than reported.

Where:

Ipconazole cc
- Peak area = 41000
- Y-intercept = 469.401
- Slope = 90655.3

Ipconazole ct
- Peak area = 15193
- Y-intercept = 901.921
- Slope = 396768

The concentration of each individual isomer at instrument was determined by substituting the resulting analyte peak area response into the regression equation above. Using the values above, the concentration in the final sample solution was calculated as:

$$\text{Ipconazole cc Concentration at instrument } (\mu g/L)\text{: } = \frac{41000 - (469.401)}{90655.3}$$

$$\text{Ipconazole cc Concentration at instrument } (\mu g/L)\text{: } = 0.44708$$

$$\text{Ipconazole ct Concentration at instrument } (\mu g/L)\text{: } = \frac{15193 - (901.921)}{396768}$$

$$\text{Ipconazole ct Concentration at instrument } (\mu g/L)\text{: } = 0.036018$$

The residue concentrations (µg/L) of Ipconazole cc and Ipconazole ct isomers in the fortified water recovery sample were determined separately as the products of the at instrument solution concentrations determined above and the overall method dilution factor as follows:

$$\text{Ipconazole cc/ct Concentrations in µg/L} = \text{Concentration at Instrument} \times \frac{(\text{Final Volume})}{(\text{Initial Volume})}$$

Where: Initial Volume= 20.0 mL
Final Volume = 2.00 mL

Using the at instrument measured concentrations (µg/L) from above, the concentrations of Ipconazole cc and Ipconazole ct isomers in the water sample were calculated as follows:

Ipconazole cc Concentration in sample (µg/L) = 0.44708 x 0.100
Ipconazole cc Concentration in sample (µg/L) = 0.044708

Ipconazole ct Concentration in sample (µg/L) = 0.036018 x 0.100
Ipconazole ct Concentration in sample (µg/L) = 0.0036018

The individually measured isomer residue in sample concentrations above were summed together for the determination of the total Ipconazole TG test substance concentration in the water sample as shown below:

Total calculated Ipconazole TG concentration (µg/L) = 0.044708 + 0.0036018
= 0.0483098

The percent recovery was determined by dividing the concentration of the total Ipconazole TG analyte recovered in the fortified sample – average control residue (µg/L), if measured, by the nominal concentration added as shown below:

$$\text{Recovery (\%)} = \frac{\text{µg/L Found - average control residue.}}{\text{µg/L Added}} \times 100$$

For the above 0.0500 µg/L fortified sample, the percent recovery of Ipconazole TG was calculated as:

$$\text{Recovery (\%)} = \frac{0.0483098 \text{ µg/L} - 0.00 \text{ µg/L Found}}{0.0500 \text{ µg/L Added}} \times 100$$

Recovery (%) = 96.6%

This same calculation procedure was applied for the confirmation analyses of Ipconazole for this study. Alternate regression analysis fits may be utilized as dictated by data trends.