## STUDY TITLE

**Independent Laboratory Validation of the Residue Analytical Method for the Determination of Residues of Pyraclonil and Metabolites Amidepyraclonil, M-1, and M-11 in Soil, Sediment, and Water by LC-MS/MS (EAG Study Number: 85901)**

## GUIDELINE REQUIREMENTS

US EPA Ecological Effects Test Guidelines, OCSPP 850.6100

# I. SUMMARY

Nichino America, Inc. contracted Golden Pacific Laboratories, LLC (GPL) in Fresno, California, to conduct an Independent Laboratory Validation. The objective of this study was to validate the analytical method contained in the draft validation report (provided by Exponent on the behalf of Nichino) entitled "Method Validation for the Determination of Residues of Pyraclonil and three metabolites (Amidepyraclonil, M-1 and M-11) in Soil, Soil-sediment, and Water by LC-MS/MS " (EAG Study Number 85901). The analysis was validated for the determination of pyraclonil, amidepyraclonil, M-1, and M-11 in soil, sediment, and water during the first method trial with a minor method modification. The analytical method was validated to demonstrate method ruggedness and to meet US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 Test Guidelines requirements for independent laboratory method validation. The study was conducted under EPA's Good Laboratory Practice Standards (GLPs) 40 CFR Part 160. The study protocol can be found in Appendix A for further information about the design of the study.

**Independent Laboratory Validation**

The analytical method was validated for all analytes on all three matrix types during the first method trial for each matrix type. However, the first time the samples were injected for analysis, the resulting data produced did not meet acceptance criteria due to an issue with the syringe filtration step in the method. A minor modification was made and each of the analytical sets was re-injected resulting in acceptable results.

One control sample was used for each matrix in this study. All three of the control samples were received from EAG Labs frozen on dry ice. The soil sample was from Arkansas, and the sediment and water samples were from California; all samples were sourced from an ongoing aquatic field dissipation study.

There was no response in the reagent blank samples in the chromatograms corresponding to the retention of pyraclonil and M-1. However, a small chromatographic interference was observed for amidepyraclonil (333.1/316.1 m/z transition ion pair) when the sample sets were first injected and upon re-analysis of the water analytical set. This interference was not observed in the 333.1/253.2 m/z transition ion pair indicating that it was not due to a contamination of amidepyraclonil. The observed interference peak was no more than approximately one-third of the height of the Limit of Detection (LOD) level standard in the passing water analytical set and as a result, did not affect recoveries. Additionally, there was a large chromatographic interference observed for M-11 (317.1/169.0 m/z transition ion pair) in the original injection sets. After the method was modified by replacing syringe filtration with ultra-centrifugation the samples were re-injected, and the interference was no longer present. The interference was determined to have originated from the polypropylene syringe filters used to filter the sample extracts.

There was no response in the control matrix samples in the chromatograms corresponding to the retention of pyraclonil, amidepyraclonil, M-1, and M-11 (other than those described in the reagent blanks above).

Control (untreated) soil and sediment samples were analyzed using the provided analytical

method. Soil and sediment samples were extracted twice with acetonitrile/0.1M hydrochloric acid (*aq*) (4:1, v/v) and were decanted into a 250 mL graduated mixing cylinder. The combined extracts were brought up to volume and syringe-filtered through a 0.2 µm, 25 mm, polypropylene syringe filter. The resulting filtered extract was vialed and submitted for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Two transition ion pairs were quantitated for each of the analytes. After the initial filtered extracts were analyzed, it was determined that the analytes were retained on the filter. As a result, the extracts were ultra-centrifuged and the supernatant was vialed. The supernatant was analyzed and resulted in acceptable results.

Control (untreated) water samples were analyzed using the provided analytical method. Water samples were extracted with 0.2% formic acid in acetonitrile. The extract was syringe-filtered through a 0.2 µm, 25 mm, polypropylene syringe filter. The resulting filtered extract was vialed and submitted for analysis by LC-MS/MS. Two transition ion pairs were quantitated for each of the analytes. After the initial filtered extracts were analyzed, it was determined that the analytes were retained on the syringe filter and as a result, extracts were ultra-centrifuged as described previously. The supernatant was analyzed and resulted in acceptable results.

The method was validated at the Limit of Quantitation (LOQ) and at 10x LOQ (1 ppb and 10 ppb in soil and sediment and 0.1 and 1 µg/L in water) for the detection of pyraclonil, amidepyraclonil, M-1, and M-11.

## II. MATERIALS

### A. Equipment

The equipment that was used is listed below:

- Balance, Analytical, Mettler Toledo XS204
- Balance, Top Loading, 0.5-3100 g, Mettler Toledo PB 3002-S
- Centrifuge tubes, polypropylene: 50 mL
- Centrifuge tubes, polypropylene, micro-centrifuge: 1.5 mL
- Nalgene bottles, HDPE: 250 mL
- Syringe Filter, polypropylene, 0.2 µm, 25 mm (these were not used to generate the acceptable results), VWR Catalog Number 28145-483
- Glass vials, clear with screw-top: 8 mL
- Vortex Mixer, VWR VX-2500 Multi-Tube Vortexer
- Volumetric flasks, glass: 10 and 100 mL
- Bottles, amber glass with Teflon lined cap: 30 and 120 mL
- Volumetric glass pipette: various sizes
- Graduated Cylinders: various volumes
- Graduated Mixing Cylinders: 250 mL
- Disposable Pasteur pipettes, glass
- Repeating Pipette, Eppendorf Stream
- Rainin Air Displacement Pipette: L-100 and L-1000
- HPLC vials, clear glass: 1 mL
- Centrifuge, Eppendorf Multipurpose Centrifuge 5810
- Platform Shaker, Variable Speed Eberbach
- Column Heater, Hot Sleeve™ with paired PTC050 control unit
- Sciex Triple Quad 6500+ MS/MS with Shimadzu LC-20AD HPLC pumps, SIL-20AC HT autosamplers, and Rheodyne Switching Valves with MPX™ Driver Version 1.2

### B. Reagents and Standards

The following chemicals were used:

| Chemical | Grade | Manufacturer | Distributer | Part No |
|---|---|---|---|---|
| Acetonitrile | Optima | Fisher | Fisher | A996-4 |
| Formic Acid (>99.5%) | ACS | Fisher | Fisher | A117-50 |
| Hydrochloric Acid | ACS | EMD | VWR | EMD-HX0603-3 |
| Methanol | ChromAR | Macron | VWR | MK304110 |
| Water | HPLC | Fisher | Fisher | W5-4 |

#### a. Preparation of Reagent Solutions:

*0.1% Formic Acid in Acetonitrile/Water (50:50, v/v):*
Prepared by combining 4 mL of concentrated formic acid, 2000 mL of acetonitrile, and 2000 mL of HPLC-grade water and mixing well.

*Acetonitrile/0.1 M HCl (aq) (4:1, v/v):*
Prepared by combining 3200 mL of acetonitrile, 800 mL of HPLC-grade water, and 6.61 mL of concentrated hydrochloric acid and mixing well. Alternate equivalent volumes were also used.

*0.2% Formic Acid in Acetonitrile/Water:*
Prepared by combining 0.5 mL of concentrated formic acid and 250 mL of HPLC-grade water and mixing well.

*Mobile Phase A:*
*0.1% Formic Acid in Acetonitrile*: Prepared by adding 1 mL of concentrated formic acid to 1000 mL of acetonitrile and mixing well.

*Mobile Phase B:*
*0.1% Formic Acid in Water*: Prepared by adding 1 mL of concentrated formic acid to 1000 mL of HPLC-grade water and mixing well.

*Needle Wash:*
*Acetonitrile/Methanol/Water (1:1:1, v/v/v)*: Prepared by combining 150 mL of acetonitrile, 150 mL of methanol, and 150 mL of HPLC-grade water and mixing well.

2. Reference Substances

All reference substances were received cold, and in good condition along with their Certificate of Analysis (COA) and Safety Data Sheets (SDS) on June 14, 2018 from EAG Labs, Columbia, MO. A copy of the certificate of analysis (COA) for each reference substance is located in Appendix B of this report. The following table contains detailed information on each analytical reference substance used in this study.

| Analytical Standard | Description | Lot # | CAS Number | Purity (%) | Expiration Date |
|---|---|---|---|---|---|
| Pyraclonil | White/small pieces | MN11114 | 158353-15-2 | 99.63 | Mar. 31, 2021 |
| Amidepyraclonil | White/small pieces | AE C630824 00 1B99 0001 | NA | 99.5 | Sep. 8, 2018 |
| M-1 | Pale yellow solid | yk120618 | 158352-02-4 | 100 | Sep. 8, 2018 |
| M-11 | White solid | AE C635049 00 1B98 0001 | NA | 99.0 | Sep. 8, 2018 |

Upon receipt, the neat standards were stored refrigerated (4 °C ± 5 °C).

3. Preparation of Standard Solutions

The reference substances were used in the preparation of the fortification and calibration solutions. Preparation and dilution data forms pertaining to the stock solutions, spiking solutions, and calibration standards are located in the raw data. All solutions were stored refrigerated when not in use.

The storage units that stored the reference substances, stock solutions, spiking solutions, and calibration standards were temperature monitored. Temperature records showing weekly temperature ranges are located in the raw data package.

**a. Stock Solutions**

Solutions of pyraclonil, amidepyraclonil, M-1, and M-11 were prepared individually in acetonitrile for use as stock solutions.

On August 1, 2018, between 10 and 11 mg of each reference substance was individually weighed directly into a 100-mL volumetric flask and diluted to the mark with acetonitrile. The resulting stock solutions contained approximately 100 µg/mL of each individual analyte when corrected for purity. The table below describes the details for each weighing.

| Stock Solution Preparation Details | | | | |
|---|---|---|---|---|
| **Analytical Reference Substance** | **Net Weight (g)** | **Weight Corrected for Purity (mg)** | **Final Volume (mL)** | **Final Conc. (µg/mL)** |
| Pyraclonil | 0.0109 | 10.85967 | 100 | 109 |
| Amidepyraclonil | 0.0106 | 10.547 | 100 | 106 |
| M-1 | 0.0102 | 10.2 | 100 | 102 |
| M-11 | 0.0105 | 10.395 | 100 | 104 |

**b. Intermediate Solution**

A 1-mL aliquot of each of the four stock solutions was added to a 10 mL volumetric flask and the flask was brought to volume with acetonitrile, resulting in a solution that contained approximately 10 µg/mL mixed solution containing all four analytes (Solution A). Solution A was used to prepare the 1 µg/mL fortification solution.

**c. Fortification Solutions**

A 1-mL aliquot of Solution A was diluted to 10 mL with acetonitrile,

resulting in a solution that contained approximately 1 µg/mL of all four analytes (Solution B). This solution was used to fortify the 10x LOQ soil and sediment samples.

Further, a 1-mL aliquot of Solution B was diluted to 10 mL with acetonitrile, resulting in a solution that contained approximately 100 ng/mL of all four analytes (Solution C). This solution was used to fortify the LOQ soil and sediment samples and the 10x LOQ water samples.

Subsequently, a 1-mL aliquot of Solution C was diluted to 10 mL with acetonitrile, resulting in a solution that contained approximately 10 ng/mL of all four analytes (Solution D). This solution was used to fortify the LOQ water samples.

The fortification solutions were stored refrigerated when not in use.

**d. Calibration Standards**

All calibration standards were diluted into 0.1% formic acid in acetonitrile/water (50:50, v/v). An aliquot (0.2 mL) of the intermediate solution above (Solution A) was diluted to 100 mL with 0.1% formic acid in acetonitrile/water (50:50, v/v) to prepare the calibration curve intermediate solution at a nominal concentration of 20 ng/mL (Solution E). The calibration standards were prepared by diluting the solutions as listed in the table as follows into volumetric flasks:

| Initial Solution ID | Volume of Solution (mL) | Final Volume (mL) | Final Solution ID | Nominal Standard Concentration (ng/mL) |
|---|---|---|---|---|
| E | 1 | 10 | F | 2 |
| E | 0.5 | 10 | G | 1 |
| E | 0.25 | 10 | H | 0.5 |
| E | 0.1 | 10 | I | 0.2 |
| E | 0.05 | 10 | J | 0.1 |
| G | 0.5 | 10 | K | 0.05 |
| G | 0.25 | 10 | L | 0.025 |

## C. Safety and Health

Material Safety Data Sheets (MSDS) and/or Safety Data Sheets (SDS) were available. Proper personal protective equipment was worn during the execution of this method. Staff avoided breathing chemical vapor and avoided chemical contact with eyes and skin. Caution was used when handling concentrated formic acid and

hydrochloric acid. There were no other procedural steps that required special precautions to avoid safety or health hazards.

## III. METHODS

### A. Principle of Analytical Method

The analysis of soil, sediment, and water was performed according to the analytical method contained in the draft validation report entitled "Method Validation for the Determination of Residues of Pyraclonil and three metabolites (Amidepyraclonil, M-1 and M-11) in Soil, Soil-sediment, and Water by LC-MS/MS" (EAG Study Number 85901). The limit of quantitation (LOQ) was defined as 1 ppb for soil and sediment, and 0.1 µg/L for water. The limit of detection (LOD) was 0.25 ppb for soil and sediment, and 0.05 µg/L for water.

The method validations for soil, sediment, and water were performed on September 4, 2018 with an additional method modification applied on September 7, 2018. All samples for each validation were extracted in one analytical set.

The soil and sediment sets consisted of one reagent blank sample, two control samples, five LOQ laboratory fortification samples and five 10x LOQ laboratory fortification samples. The water set consisted of one reagent blank sample (HPLC grade water), two control samples, five LOQ laboratory fortification samples, and five 10x LOQ laboratory fortification samples. Prior to extraction, a unique laboratory code designation was assigned by GPL to each sample. The laboratory code consisted of the last three digits of the GPL study number; the sample set designation and a sample number (e.g., 770LV01-1).

Detailed method flow charts for the ILV trials can be found in Appendix C and the draft analytical method report that was followed for this study can be found in Appendix D.

1. Soil and Sediment

Soil and sediment samples were extracted twice with acetonitrile/0.1M hydrochloric acid (*aq*) (4:1, v/v) using a platform shaker and were decanted into a 250 mL graduated mixing cylinder. The combined extracts were brought up to volume and syringe-filtered through a 0.2 µm, 25 mm, polypropylene syringe filter. The resulting filtered extract was vialed and submitted for analysis by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Two transition ion pairs were quantitated for each of the analytes. After the initial filtered extracts were analyzed, it was determined that the analytes were retained on the filter. As a result, extracts were ultra-centrifuged instead of being filtered using syringe filters and the supernatant was vialed. The supernatant was analyzed and resulted in acceptable results.

2. Water

Water samples were extracted with 0.2% formic acid in acetonitrile. The extract was syringe-filtered through a 0.2 µm, 25 mm, polypropylene syringe filter. The resulting filtered extract was vialed and submitted for analysis by LC-MS/MS. Two transition ion pairs were quantitated for each of the analytes. After the initial filtered extracts were analyzed, it was determined that the analytes were retained on the filter. As a result, the extracts were ultra-centrifuged instead of being filtered using syringe filters and the supernatant was vialed. The supernatant was analyzed and resulted in acceptable results.

## B. Soil and Sediment Analytical Procedure

1. Control Matrixes

The control samples were received from EAG Labs frozen on dry ice. The soil sample was from Arkansas, and the sediment was from California; all samples were sourced from an ongoing aquatic field dissipation study. Both samples were stored in a freezer set to maintain ≤ -10 °C (frozen) when not in use.

2. Preparation of Samples

Sub-samples (20 g ± 0.1 g) of the control soil or sediment were measured into 250 mL Nalgene bottles.

3. Fortifications

Samples were fortified at the LOQ (1 ppb) or 10x the LOQ (10 ppb). Fortifications were performed using Rainin air displacement pipettes to fortify the 20-g samples directly as follows:

| Fortification Level | Amount and Concentration of Pyraclonil/Amidepyraclonil/ M-1/M-11 Spiking Solution Used |
|---|---|
| LOQ (1 ppb) | 200 µL 109/106/102/104 ng/mL |
| 10x LOQ (10 ppb) | 200 µL 1.09/1.06/1.02/1.04 µg/mL |

4. Extraction

After fortification, an aliquot (100 mL) of acetonitrile/0.1 M HCl (*aq*) (4:1, v/v) was added to each soil or sediment sample using a 100-mL graduated

cylinder. The samples were capped and shaken on a platform shaker for 20 minutes at approximately 200 rpm. The samples were then centrifuged at a setting of 3000 rpm and 10 minutes. Following centrifugation, the supernatants were decanted into a 250-mL graduated mixing cylinder. The solids were re-extracted with another 100-mL aliquot of acetonitrile/0.1 M HCl (*aq*) (4:1, v/v), capped, and shaken on a platform shaker for 20 minutes at approximately 200 rpm. The samples were centrifuged at a setting of 3000 rpm and 10 minutes. Following centrifugation, the supernatants were decanted into the same 250-mL graduated mixing cylinder. The combined extracts were brought up to 200 mL with acetonitrile/0.1 M HCl (*aq*) (4:1, v/v). Since a large portion of the sediment samples were water, the extraction volumes came out between 202 mL and 205 mL. A portion of each of the extracts was then syringe filtered into an 8-mL glass vial using a 0.2 µm, 25 mm, polypropylene syringe filter. Subsequently, the filtered extracts were vialed and submitted for analysis by LC-MS/MS.

5. Extraction Modification

In order to produce acceptable results after the problems observed from the first injections, aliquots (~ 1 mL) of the final extracts (before filtration) were transferred into centrifuge tubes (~ 1.5 mL). Aliquots were centrifuged at a setting of 14000 rpm and 5 minutes. The supernatants were subsequently vialed and submitted for analysis by LC-MS/MS. This method modification produced acceptable results.

## C. Water Analytical Procedure

1. Control Matrixes

The control samples were received from EAG Labs frozen on dry ice. The water sample was from California, sourced from an ongoing aquatic field dissipation study. The control sample was stored refrigerated when not in use.

2. Preparation of Samples

Sub-samples (10 mL) of the control water were measured into 50-mL plastic centrifuge tubes.

3. Fortifications

Samples were fortified at the LOQ (1 ppb) or 10x the LOQ (10 ppb). Fortifications were performed using Rainin air displacement pipettes to fortify the 10-mL samples directly as follows:

| Fortification Level | Amount and Concentration of Pyraclonil/Amidepyraclonil/M-1/M-11 Spiking Solution Used |
|---|---|
| LOQ (0.1 µg/L) | 100 µL 10.9/10.6/10.2/10.4 ng/mL |
| 10x LOQ (1 µg/L) | 100 µL 109/106/102/104 ng/mL |

4. Extraction

An aliquot (10 mL) of 0.2% formic acid was added to each water sample. The samples were capped and then mixed on a multi-tube vortexer for approximately 1 minute. A portion of each of the extracts was then syringe filtered into an 8-mL glass vial using a 0.2 µm, 25 mm, polypropylene syringe filter. Subsequently, the filtered extracts were vialed and submitted for analysis by LC-MS/MS.

5. Extraction Modification

In order to produce acceptable results after the problems observed from the first injections, aliquots (~ 1 mL) of the final extracts (before filtration) were transferred into centrifuge tubes (~ 1.5 mL). Aliquots were centrifuged at a setting of 14000 rpm and 5 minutes. The supernatants were subsequently vialed and submitted for analysis by LC-MS/MS. This method modification produced acceptable results.

## D. Instrumentation

Instrument: Sciex Triple Quad™ 6500+ LC-MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SIL-20AC HT Autosamplers, Rheodyne Switching Valves, MPX™ Driver Ver. 1.2, and Analyst Data System Ver. 1.6

HPLC Column: Waters Acquity UPLC® HSS T3
50 x 2.1 mm, 1.8 µm
Part #186003538
Serial #02103818618449

Guard Column: Waters Acquity UPLC® HSS T3 VanGuard™
5 x 2.1 mm, 1.8 µm
Part #186003976

Column Heater: Hot Sleeve, 10L or 15L

Data System: Analyst Data System version 1.6, AB Sciex

Mobile Phases:

A) 0.1% formic acid in acetonitrile
B) 0.1% formic acid in water

Flow Rate: 0.5 mL/minute
Run Time: 7.0 minutes
Injection Volume: 10 µL
Gradient Program:

| Time (minutes) | %A | %B |
|---|---|---|
| 0.0 | 5 | 95 |
| 0.5 | 5 | 95 |
| 5.0 | 95 | 5 |
| 6.0 | 95 | 5 |
| 6.01 | 5 | 95 |
| 7.0 | 5 | 95 |

Column Heater: 40 ° C

Retention Time:

Pyraclonil: ~3.8 minutes
Amidepyraclonil: ~3.2 minutes
M-1: ~3.6 minutes
M-11: ~3.9 minutes

*Note: In the raw data and report chromatograms, 0.10 minutes must be added to the retention time on the chromatogram to adjust for the mass spectrometer offset.*

Mass Spectrometer Parameters (operated in LC-MS/MS mode):

| AB Sciex API 6500+ Acquisition Parameters (TurboIonSource, ESI interface, MRM mode, positive mode, Unit/Unit Resolution) | | | | | | |
|---|---|---|---|---|---|---|
| **Analyte** | **Q1 (m/z)** | **Q3 (m/z)** | **Dwell (msec)** | **DP** | **CE** | **EP** |
| Pyraclonil | 315.2 | 169.1 | 40 | 60 | 37 | 10 |
| Pyraclonil | 315.2 | 241.2 | 10 | 60 | 30 | 10 |
| Amidepyraclonil | 333.1 | 253.2 | 40 | 40 | 30 | 10 |
| Amidepyraclonil | 333.1 | 316.1 | 10 | 40 | 15 | 10 |
| M-1 | 277.2 | 250.2 | 40 | 70 | 26 | 10 |
| M-1 | 277.2 | 214.2 | 10 | 70 | 31 | 10 |
| M-11 | 317.1 | 169.0 | 40 | 60 | 38 | 6 |
| M-11 | 317.1 | 241.1 | 10 | 60 | 31 | 6 |

| Parameter | Setting |
|---|---|
| CUR | 40 |
| GS1 | 40 |
| GS2 | 40 |
| IS | 2500 |
| TEM | 500 |
| CAD | 10 |
| CXP | 10 |

The reference method used the transition ion pair 315/99 m/z as the second ion pair for pyraclonil. The 315/241 m/z transition ion pair produced a greater signal-to-noise ratio, and was quantitated in place of the 315/99 m/z transition ion pair for the purposes of this study.

## E. Potential Interferences

There was no response in the reagent blank samples in the chromatograms corresponding to the retention of pyraclonil and M-1. However, a small chromatographic interference was observed for amidepyraclonil (333.1/316.1 m/z transition ion pair) in the water, soil, and sediment sets when the sample sets were first injected and upon re-analysis of the water analytical set. Also, the interference was not observed in the 333.1/253.2 m/z transition ion pair indicating that it was not a contamination of amidepyraclonil. The inference was no more than approximately one-third of the height of the LOD level standard in the passing water analytical set and as a result, did not affect recoveries. The source of this minor interference is unknown.

1. Matrix Interference

The detection technique is highly selective for this method. No interferences arising from co-eluting compounds from the water, soil, or sediment samples was observed.

2. Reagent and Solvent Interference

High purity solvents and reagents were used for this assay. Other than the amidepyraclonil interference listed above (with unknown origin), no interferences were observed as a result of the reagent and solvents.

3. Labware Interference

This method uses mostly disposable labware. However, there was a large

chromatographic interference observed for M-11 (317.1/169.0 transition ion pair) in the original injection sets. After the method was modified by replacing the filtration step (using syringe filters) with ultra-centrifugation the samples were re-injected, this interference was no longer present. The interference was determined to have come from the 0.2 µm, 25 mm polypropylene syringe filter (VWR cat# 28145-483). Further information regarding the method modification is described in detail elsewhere in this report.

No other interferences from the labware use were observed.

## F. Confirmatory Techniques

The independent laboratory validation sets were run by LC-MS/MS with monitoring of two ion transition pairs. As this method is highly selective, no additional confirmatory technique was used.

## G. Time Required for Analysis

1. Soil or Sediment Sample Analysis

Approximately 4 hours was required for one person to prepare an analysis set of soil or sediment samples from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed immediately and required approximately 3 to 4 hours. An additional 2 hours was spent on data calculation per analysis set. At most, two calendar days are needed to prepare an analysis set and to calculate and tabulate the data (when samples are analyzed overnight).

2. Water Sample Analysis

Approximately 30 minutes was required for one person to prepare an analysis set of water samples from the time samples were prepared to LC-MS/MS analysis. Automated LC-MS/MS analysis was performed immediately and required approximately 3 to 4 hours. An additional 2 hours was spent on data calculation per analysis set. At most, two calendar days are needed to prepare an analysis set and to calculate and tabulate the data (when samples are analyzed overnight).

## H. Modification or Potential Problems

1. Description of Problems

There were two problems encountered that resulted in one necessary modification to the method for both the water and soil/sediment analysis procedures. The two problems were the adsorption of the target analytes,

which caused low recoveries, and an observed interference with quantitation of M-11 (317.1/169.0 transition ion pair). Both problems were related to the polypropylene syringe filter used in this study.

The water, soil, and sediment validations were all conducted on the same day. Once the results were tabulated, it was determined that the data were consistent however, they did not meet acceptance criteria

Additionally, there was a large chromatographic interference observed for M-11 (317.1/169.0 transition ion pair) in the original injection sets.

Since the water, soil, and sediment results were all similar and showed the same interference for M-11, it became apparent that the method issue was not unique between the water and the soil and sediment method procedure. Upon examination for similarities, it was determined that the syringe filtration step most likely contributed to recovery and interference problems.

2. Filter Tests

Filtration tests were completed on a 10x LOQ sample from the water and soil extraction sets to confirm that the filtration step was the source of the problem. The data from these tests are summarized below.

It was concluded that the filtration step was causing the low recovery of each of the analytes. Alternative filter types and sizes were also evaluated. A suitable substitute filter was not found. As a result, an extra centrifugation step was added to the method in lieu of the filtration step in order to remove suspended particulate matter from the final extract for injection.

The filtration tests also confirmed that the presence of the interference peak in the M-11 (317.1/169.0 transition ion pair) samples was due to the filter.

3. Resulting Modification

The method modification produced acceptable results.

## I. Methods of Calculation

Analyst Chromatography Data System version 1.6, a product of AB Sciex, was used to acquire, integrate and calculate the concentrations pyraclonil, amidepyraclonil, M-1, and M-11 as ng/mL using the linear regression function with 1/x weighting. The calibration was not forced through the origin. For the regression calculations, concentration was designated as the independent variable and plotted on the x-axis. Peak area response was designated as the dependent variable and plotted on the y-axis. From this regression curve, a slope, a correlation coefficient, and other parameters of the standard curve were calculated. Calibration standards were injected every three to five sample injections as well as at the beginning and end of the injection sequence. Seven different standard concentrations were injected within the analytical set. The concentrations (ng/mL) of each analyte detected in method validation sample extracts were interpolated from the standard calibration curve.

The concentration as µg/L of residue found in water samples was then calculated with Microsoft® Excel using the following equation:

$$\mu g/L = \frac{(ng/mL \text{ from curve}) \times (\text{Final Volume in mL}) \times 1\ \mu g \times 1000\ mL}{(\text{Sample amount in mL}) \times 1000\ ng \times 1\ Liter}$$

The concentration as ppb of residue found in soil and sediment samples was then calculated with Microsoft® Excel using the following equation:

$$ppb = \frac{(ng/mL \text{ from curve}) \times (\text{Final Volume in mL})}{(\text{Sample amount in g})}$$

Recovery of the analyte from fortified samples was calculated as follows:

$$\%\ Recovery = \frac{(\text{Measured Concentration, } \mu g/L \text{ or ppb}) \times 100}{(\text{Theoretical Concentration, } \mu g/L \text{ or ppb added})}$$

An example calculation for water for an M-11 laboratory fortification (317.1/169.0 m/z transition ion pair) in set 770ILV01, sample 770ILV01-10 10x LOQ sample fortified at 1.04 µg/L, is as follows:

*standard curve equation:* $y = 1.43 \times 10^6 (x) + 725$

*where x = M-11 concentration in ng/mL and*

*y = peak response = 790216.8*

*M-11 concentration from the curve = 0.552 ng/mL*

$$\mu g/L = \frac{(0.552\ ng/mL\ M\text{-}11) \times (20\ mL) \times 1\mu g \times 1000\ mL}{(10\ mL) \times 1000\ ng \times 1\ Liter} = 1.10\ \mu g/L$$

$$\% \text{ recovery} = \frac{1.10\ \mu g/L}{1.04\ \mu g/L} \ X\ 100 \quad = \quad 106\%$$

An example calculation for soil for a pyraclonil laboratory fortification (315.2/169.1 m/z transition ion pair) in set 770ILV02, sample 770ILV02-6 LOQ sample fortified at 1.09 ppb, is as follows:

*standard curve equation:* $y = 1.34 \times 10^6 (x) + 1.82 \times 10^3$

*where x = pyraclonil concentration in ng/mL and*

$y$ = *peak response = 136305.5*

*pyraclonil concentration from the curve = 0.100 ng/mL*

$$ppb = \frac{(0.100\ ng/mL\ pyraclonil) \times (200\ mL)}{(20.03\ g)} = 0.999\ ppb$$

$$\% \text{ recovery} = \frac{0.999\ ppb}{1.09\ ppb} \ X\ 100 \quad = \quad 91.7\%$$

No detectable residues were measured in any control samples. Laboratory fortification samples were not corrected for reported control responses. Rounding differences result in minor variations in values between the results obtained using the standard curve equation and peak area response above in the calculations versus those values in the report tables and raw data.

## J. Statistical Procedures

Laboratory statistical procedures included calculation of arithmetic mean, the corresponding standard deviation (where $n \geq 3$), coefficient of variation and 95% confidence interval for analyte recovery data. Linear regression analysis was applied to LC-MS/MS calibration curves for the determination of slope, y-intercept, and correlation coefficient values.

The modified method is precise and meets all precision acceptance criteria of the US EPA Ecological Effects Test Guidelines, OCSPP 850.6100 (i.e., < ± 20% at each fortification level).

**D. Limit of Detection**

As a matter of setting a threshold for reporting residue values in the reagent blank and control samples the LOD was applied at 0.25 ppb for soil and sediment, and 0.05 µg/L for water. Practical LODs were previously determined in the reference method.

**E. Limit of Quantitation**

The LOQ for pyraclonil, amidepyraclonil, M-1, and M-11 was 1 ppb in soil and sediment, and 0.1 µg/L in water.

**F. Selectivity and Specificity**

There was no apparent response in the unfortified samples in the region of the chromatograms at the retention time for pyraclonil and M-1 suggesting the method is selective for pyraclonil and M-1 in soil, sediment, and water. However, an interference of unknown origin was noted for amidepyraclonil in the water reagent blank and control method validation samples in the 333.1/316.1 m/z transition ion pair chromatograms (from the analytical set that met acceptance criteria).

Furthermore, there was a chromatographic interference observed for M-1 from the polypropylene syringe filter in the soil, sediment, and water, reagent blank, control, and fortified method validation samples in the 317.1/169.0 m/z transition ion pair chromatograms (from the analytical set that did not meet acceptance criteria). This

selectivity problem was remedied a minor method modification by ultra-centrifuging the extracts rather than filtering the extracts for LC-MS/MS analysis. The interference was not observed when the polypropylene syringe filter was not used.

The method is specific for pyraclonil, amidepyraclonil, M-1, and M-11 due to the use of two different transition ion pairs.

## G. Limitations

The method has been tested in soil from Arkansas, sediment from California, and water from California. It can be assumed that the method may be applicable to other soil, sediment, and water matrixes not tested in these validations provided successful recovery tests are conducted at relevant fortification levels.

# Appendix A

# Study Protocol

**STUDY PROTOCOL**

**Independent Laboratory Validation of the Residue Analytical Method for the Determination of Residues of Pyraclonil and Metabolites Amidepyraclonil, M-1, and M-11 in Soil, Sediment, and Water by LC-MS/MS (EAG Study Number: 85901)**

**Guideline Requirements**

USA EPA Ecological Effects Test Guidelines, OCSPP 850.6100

## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| % | percent |
| °C | degrees centigrade |
| µg/mL | microgram per milliliter |
| µL | microliter |
| µm | micrometer |
| aq. | aqueous |
| CI | Confidence Interval |
| Fort. | Fortification |
| g | gram |
| g/mol | grams per mol |
| HCl | Hydrochloric Acid |
| HPLC | High-Performance Liquid Chromatography |
| Inc. | Incorporated |
| L | Liter |
| LC-MS/MS | liquid chromatography/tandem mass spectrometry (2-stage mass analysis experiment), $MS^2$ |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| M | Molar |
| mg | milligram |
| min | minute |
| mL | milliliter |
| mm | millimeter |
| MRM | multiple reaction monitoring |
| *m/z* | mass/charge ratio |
| n | number |
| NA | Not applicable |
| ND | None detected |
| ng/mL | nanogram per milliliter |

**LIST OF ABBREVIATIONS AND SYMBOLS (continued)**

| | |
|---|---|
| No. | number |
| OCSPP | Office of Chemical Safety and Pollution Prevention |
| ppb | parts per billion |
| rpm | revolutions per minute |
| RSD | relative standard deviation (StDev / mean*100) |
| SD | Standard deviation |
| SOP | Standard Operating Procedure |
| Std. Dev. | standard deviation (determined using Excel® function STDEV) |
| UPLC | Ultra-Performance Liquid Chromatography |
| v | volt |
| v:v | Volume to volume ratio (aqueous solutions) |
| Vol. | Volume |

## 2 EQUIVALENCE STATEMENT

During the conduct of this analysis, comparable apparatus, solvents, glassware, and techniques may be substituted for those described in this method, except where specified. In the event a substituted piece of equipment or technique is used, its use will be documented in the study records.

## 3 APPARATUS AND EQUIPMENT

Due to the potential for contamination resulting from low detection limits, disposable equipment should be used when possible. If glassware is used, care should be taken to minimize the potential for contamination due to insufficient cleaning of the glassware. Common laboratory glassware and supplies are assumed to be readily available.

| | |
|---|---|
| Analytical Balances (weighing reference standards): | Mettler XP205DR (Mettler Instrument Corporation, Hightstown, NJ) |
| Top-loading Balance (weighing samples): | Mettler ML3002E (Mettler Instrument Corporation, Hightstown, NJ) |
| Centrifuge: | Sorvall Legend XTR Benchtop Centrifuge |
| HPLC-MS/MS System: | Applied Biosystems/Sciex API 6500 Q-Trap Mass Spectrometer LC-MS/MS system, with Shimadzu SIL-30ACMP Autosampler, Shimadzu LC-30AD Pumps, Shimadzu DGU-20A5R Degasser, Shimadzu CT0-20A Column Oven, and Shimadzu CBM-20A Communications Bus Module (System Controller) with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (Version 1.6.2) |
| HPLC Column: | Acquity UPLC HSS T3, 50 mm x 2.1 mm, 1.8 µm |
| Pipets (glass): | Graduated, serological, various sizes; Class-A, various sizes |
| Pipets (automatic): | Gilson – various sizes and tips (Gilson, Inc., Middletown, WI), Eppendorf repeating pipette |
| Stock Solution Containers: | Volumetric flasks, glass, Class-A, various sizes<br>Amber vials, glass, and polypropylene tubes various sizes (for storage) |
| Sample Containers: | 50-mL polypropylene centrifuge tubes<br>250-mL mixing cylinder<br>250-mL polypropylene bottles |
| Additional glassware/equipment: | graduated cylinder, various sizes<br>2-mL glass autosampler vials<br>Sonicators, shakers, vortex mixers<br>0.2 µm, 25 mm polypropylene syringe filter |

## 4 REAGENTS AND MATERIALS

Reagents are HPLC-grade or higher, except where noted. Wear proper personal protective equipment when handling chemicals and reagents. Review each chemical SDS for further safety information.

| | |
|---|---|
| Acetonitrile: | Fisher Scientific, Fairlawn, NJ<br>Honeywell Burdick & Jackson, Muskegon, MI |
| Formic Acid: | Fisher Scientific, Fairlawn, NJ, Acros Organic (a division of Fisher Scientific), concentration >98% |
| Hydrochloric Acid (HCl): | Concentrated (12.1M), Fisher Scientific, Fairlawn, NJ |
| Methanol: | Fisher Scientific, Fairlawn, NJ |
| Water: | Fisher Scientific, Fairlawn, NJ |

### 4.1 Reagents and Materials to be Prepared

Volumes may be adjusted accordingly for different quantities. Reagent solution stability was assigned based on EAG Standard Operating Procedures. Alternate expirations may be assigned as necessary based on individual laboratory standard procedures.

4:1 Acetonitrile:0.1 M HCl

Combine 3200 mL of acetonitrile, 800 mL of water and 6.61 mL of HCl. Cap and mix well. The solution is stable for 1 year when stored at room temperature.

Combine 4800 mL of acetonitrile, 1200 mL of water and 9.92 mL of HCl. Cap and mix well. The solution is stable for 1 year when stored at room temperature.

0.1% Formic Acid in Acetonitrile:Water (50:50, v:v)

Combine 4 mL of 98%+ formic acid, 2000 mL of acetonitrile and 2000 mL of water. Cap and mix well. The solution is stable for 1 year when stored at room temperature.

0.1% Formic Acid in Water

Add 20 mL of 98%+ formic acid to 20 L of water and mix well. The solution is stable for at least 3 months when stored at room temperature.

0.1% Formic Acid in Acetonitrile

Add 4 mL of 98%+ formic acid to ~4-L of acetonitrile and mix well. The solution is stable for at least 1 year when stored at room temperature.

0.2% Formic Acid in Acetonitrile

Add 2 mL of 98%+ formic acid to 1000-mL of acetonitrile and mix well. The solution is stable for at least 1 year when stored at room temperature.

0.2% Formic Acid in Acetonitrile:water (20:80, v:v)

Add 400 mL of water to 100-mL of acetonitrile. Add 1 mL of 98%+ formic acid and mix well. The solution is stable for at least 6 months when stored at room temperature.

1:1:1 Acetonitrile:Methanol:Water

Combine 4000 mL each of acetonitrile, methanol, and HPLC-grade water and mix well. The solution is stable for at least 1 year when stored at room temperature.

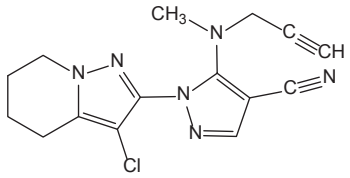
1:1:2 Acetonitrile:Methanol:Water

Combine 4000 mL each of acetonitrile and methanol with 8000 mL of HPLC-grade water and mix well. The solution is stable for at least 1 year when stored at room temperature.

## 5 REFERENCE STANDARDS

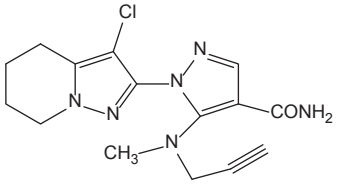
***Pyraclonil:***

Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridin-2-yl)-5-[methyl(prop-2-ynyl)amino]pyrazole-4-carbonitrile
Molecular Weight: 314.1 g/mol
Molecular Formula: $C_{15}H_{15}ClN_6$
Structural Formula:



***Amidepyraclonil:***

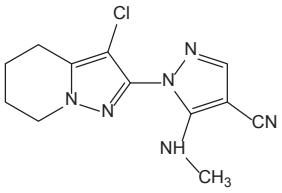
Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridin-2-yl)-5-[methyl-prop-2-ynyl)amino]-pyrazole-4-carboxamide
Molecular Weight: 332.1 g/mol
Molecular Formula: $C_{15}H_{17}ClN_6O$
Structural Formula:



***M-1:***

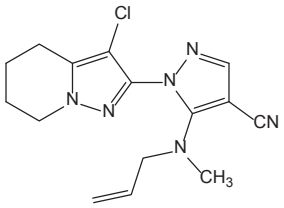
Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridin-2-yl)-5-(methylamino)-pyrazole-4-carbonitrile
Molecular Weight: 276.1 g/mol
Molecular Formula: $C_{12}H_{13}ClN_6$

Structural Formula:




***M-11:***

Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridine-2-yl)-5-[methyl(prop-2-enyl)amin]pyrazole-4-carboxnitrile
Molecular Weight: 316.1 g/mol
Molecular Formula: $C_{15}H_{17}ClN_6$
Structural Formula:




## 6 STANDARD PREPARATION

Prepare all standard solutions in HPLC-grade solvents using appropriate analytical techniques. Alternative or additional standard concentrations and volumes may be prepared as needed.

### 6.1 Stock Standard Solutions

The maximum storage interval determined for stock standard solutions was 95 days. For ease of reporting, stability for stock standard solutions prepared in acetonitrile will be documented as 3 months when stored in an amber glass bottle at 2-8 °C.

Approximately 10 mg (corrected for purity) of analytical standard is quantitatively transferred to a 100-mL volumetric flask using acetonitrile. The solution is sonicated if needed and allowed to

equilibrate to room temperature prior to being brought to volume with acetonitrile to make a stock standard solution of approximately 100 µg/mL.

### 6.2 Fortification Standard Solutions

Typically, the following concentrations of fortification standard solutions are prepared. These solutions prepared in acetonitrile have been demonstrated to be stable for between 69 and 77 days. For ease of reporting, stability for fortification solutions will be documented as 2 months when stored in an amber glass bottle at 2-8 °C.

10.0 µg/mL (mixed): Transfer 1.00 mL of each 100.0-µg/mL fortification standard solution to a 10-mL volumetric flask. Bring to volume with acetonitrile. Mix well.

1.00 µg/mL (mixed): Transfer 1.00 mL of the 10.0-µg/mL (mixed) fortification standard solution to a 10-mL volumetric flask. Bring to volume with acetonitrile. Mix well.

0.100 µg/mL (mixed): Transfer 1.00 mL of the 1.00-µg/mL (mixed) fortification standard solution to a 10-mL volumetric flask. Bring to volume with acetonitrile. Mix well.

0.0100 µg/mL (mixed): Transfer 0.100 mL of the 1.00-µg/mL (mixed) fortification standard solution to a 10-mL volumetric flask. Bring to volume with acetonitrile. Mix well.

### 6.3 Calibration Standard Solutions

Suppression or enhancement in the presence of matrix was <20% during method validation, therefore, solvent based calibration standards can be employed. Typically, the following intermediate and calibration solutions are prepared. Standard concentrations range from approximately 50% of the LOQ to approximately 4 times the expected concentration of the 10 x LOQ fortification. These solutions prepared in 0.1 % formic acid in acetonitrile:water (50:50, v:v) have been demonstrated to be stable for between 69 and 77 days. For ease of reporting, stability for fortification solutions will be documented as 2 months when stored in an amber glass bottle at 2-8 °C.

<u>Intermediate Solvent Standard Solution:</u>

20.0 ng/mL (mixed): Transfer 0.200 mL of the 10.0-µg/mL (mixed) fortification standard solution to a 100-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

Calibration Solvent Standard Solutions:

2.00 ng/mL (mixed): Transfer 1.00 mL of the 20.0-ng/mL (mixed) intermediate standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

1.00 ng/mL (mixed): Transfer 0.500 mL of the 20.0-ng/mL (mixed) intermediate standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

0.500 ng/mL (mixed): Transfer 0.250 mL of the 20.0-ng/mL (mixed) intermediate standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

0.200 ng/mL (mixed): Transfer 0.100 mL of the 20.0-ng/mL (mixed) standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

0.100 ng/mL (mixed): Transfer 0.0500 mL of the 20.0-ng/mL (mixed) standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

0.0500 ng/mL (mixed): Transfer 0.500 mL of the 1.00-ng/mL (mixed) standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

0.0250 ng/mL (mixed): Transfer 0.250 mL of the 1.00-ng/mL (mixed) standard solution to a 10-mL volumetric flask. Bring to volume with 0.1% formic acid in acetonitrile:water (50:50, v:v). Mix well.

## 7 SAMPLE ORIGINS, PREPARATION, & STORAGE

Untreated control soil, soil-sediment and water samples were transferred from the California and Arkansas trial sites for the purpose of supporting ongoing Nichino America aquatic field dissipation studies, under EAG Study Nos. 85589 and 85590, respectively. Chain of Custody documentation is included in the raw data.

Soil and soil-sediment sample preparation was performed by EAG Laboratories. Soil samples were ground using a Hammermill grinder. Dry ice was passed through to cool it before sample processing. Each frozen composite sample was then ground in the presence of enough dry ice to keep the sample frozen.

After grinding, the samples were placed in pre-labeled containers and the dry ice was allowed to sublime in a freezer over several days. The sample grinding equipment was cleaned after each sample was processed to avoid contamination.

The control soil and soil-sediment samples from both California and Arkansas were processed and stored frozen prior to analysis.

## 8 SAMPLE FORTIFICATION & EXTRACTION

### 8.1 Soil/Soil-sediment Sample Fortification

1. Weighed 20 (± 0.10) g of homogenized sample into a 250-mL polypropylene bottle.

2. Fortified applicable samples with the appropriate amount of standard solution.

### 8.2 Soil/Soil-sediment Sample Extraction

1. Added 100 mL of acetonitrile:0.1M HCl (aq) (4:1, v:v) to the sample using a 100-mL graduated cylinder.

2. Securely capped the sample bottle and placed onto a mechanical shaker for ~20 minutes at low speed.

3. Centrifuged the sample at ~3000 rpm for ~10 minutes.

4. Decanted the supernatant into a 250-mL graduated mixing cylinder.

5. Repeated Steps 1 through 4, for a total of 2 x 100 mL of acetonitrile:0.1M HCl (aq) (4:1, v:v), combining the supernatant in the same 250-mL graduated mixing cylinder.

6. Diluted the combined sample extract to the 200 mL mark on the mixing cylinder with acetonitrile:0.1M HCl (aq) (4:1, v:v) and transferred into a 250-mL polypropylene bottle.

7. Filtered a portion of the extract of each sample (or dilution thereof) into a 2-mL glass vial using a 0.2 µm, 25 mm polypropylene syringe filter.

8. Vialed a portion of each standard into a 2-mL glass vial.

9. Submitted samples and standards for UPLC-MS/MS analysis.

### 8.3 Water Sample Fortification

1. Allowed sample to warm to room temperature. Aliquoted 10 (± 0.10) mL of sample into a 50-mL polypropylene tube.

2. Fortified applicable samples with the appropriate amount of standard solution.

### 8.4 Water Sample Extraction

1. Added 10 mL of 0.2% Formic acid in acetonitrile to the sample.

2. Securely capped and vortex mixed the sample for ~1 minute.

3. Filtered a portion of the extract of each sample (or dilution thereof) into a 2-mL glass vial using a 0.2 µm, 25 mm polypropylene syringe filter.

4. Vialed a portion of each standard into a 2-mL glass vial.

5. Submitted samples and standards for UPLC-MS/MS analysis.

Extract stability was demonstrated during the course of this study by injecting aged fortification extracts and evaluating the percent recoveries. The details of this analysis are presented in Table 8 through Table 11 and a comparative summary is presented in Table 12.

## 10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

The column and conditions stated below have been satisfactory for the matrices being analyzed. The specific column packing, mobile phase, column temperature, and flow rate listed are typical conditions for this analysis. Alternate columns may be used depending on the need to resolve analyte and/or interfering responses. Specific conditions used will be noted with each chromatographic run and will not otherwise be documented.

### 10.1 Operating Conditions

Typical UPLC conditions used for this analysis were as follows:

Operating Conditions

| | |
|---|---|
| Instrument: | Shimadzu UPLC system |
| | Applied Biosystems/Sciex API 6500 Q-Trap Mass Spectrometer LC-MS/MS system, with Shimadzu SIL-30ACMP Autosampler, Shimadzu LC-30AD Pumps, Shimadzu DGU-20A5R Degasser, Shimadzu CT0-20A Column Oven, and Shimadzu CBM-20A Communications Bus Module (System Controller) with Applied Biosystems/MDS Sciex Analyst Software for data collection and system control (Version 1.6.2) |
| UPLC Column: | Acquity UPLC HSS T3, 50 mm x 2.1 mm, 1.8 um |
| Mobile Phase: | Fisher water, Fisher methanol, Fisher acetonitrile, and Fisher Formic Acid |
| | Component A: 0.1% Formic Acid (aq) |
| | Component B: 0.1% Formic Acid in Acetonitrile |

| | | | | |
|---|---|---|---|---|
| Needle Rinse: | 1:1:1 Acetonitrile:Methanol:Water<br>1:1:2 Acetonitrile:Methanol:Water | | | |
| Gradient: | Time (min.) | % A | % B | |
| | 0.50 | 95 | 5 | |
| | 5.00 | 5 | 95 | |
| | 6.00 | 5 | 95 | |
| | 6.01 | 95 | 5 | |
| | 7.00 | stop | | |
| Flow Rate: | 0.500 mL/min | | | |
| Interface: | TIS (turbo ion spray) | | | |
| Ionization Mode: | Positive (+) | | | |
| Acquisition Mode: | MRM | | | |
| Resolution: | Q1 – unit, Q3 – unit (Note: Unit equivalent to medium) | | | |
| Source Temperature: | 600 °C | | | |
| Curtain Gas: | Nitrogen @ setting of "10" | | | |
| Collision Gas: | Nitrogen @ setting of "High" | | | |
| Transitions Monitored: | Analyte | Q1 | Q3 | CE,*v* |
| | Pyraclonil | 315<br>315<br>315 | 169<br>241<br>99 | 37 (quantitation)<br>30 (confirmation)<br>67 (confirmation) |
| | Amidepyraclonil | 333<br>333<br>333 | 253<br>316<br>288 | 25 (quantitation)<br>15 (confirmation)<br>15 (confirmation) |
| | M-1 | 277<br>277<br>277<br>277 | 250<br>214<br>182<br>185 | 26 (quantitation)<br>31 (confirmation)<br>43 (confirmation)<br>35 (confirmation) |
| | M-11 | 317<br>317<br>317 | 169<br>241<br>275 | 38 (quantitation)<br>32 (confirmation)<br>47 (confirmation) |
| Injection Volume: | 10 µL | | | |
| Column Temperature: | 40 °C | | | |
| Retention Time: | Pyraclonil | | ~3.43 minutes | |
| | Amidepyraclonil | | ~2.80 minutes | |
| | M-1 | | ~3.21 minutes | |
| | M-11 | | ~3.62 minutes | |

### 10.2 Sample Analysis

Prepare a standard curve by injecting constant volumes of standard solutions (at least 7 concentrations). Use constant volume injections for sample extracts as well. Soil and soil-sediment dilutions are prepared in 0.2% Formic Acid in Acetonitrile:water (20:80, v:v). Calibration standards should be injected intermixed with test samples before and after every

1-4 samples in each analytical set. A typical analytical run would consist of at least seven calibration standard concentrations ranging from approximately 50% of the LOQ to approximately 4 times the expected concentration of the 10 x LOQ fortification, a procedural control (non-fortified sample), a minimum of two fortified procedural controls (one of which must be at the LOQ), and associated samples.

### 10.3 Assay Time

The typical analytical run requires approximately 4 hours for water and 8 hours for soil/soil sediment to extract/purify and prepare a set of 14 samples for UPLC-MS/MS analysis, followed by approximately 3 hours of instrumental analysis.

Acceptable stopping points to allow for the continuation of a set the following day were not assessed and should be assessed as needed.

## 11 CALCULATIONS

Calculations for instrumental analysis are conducted using a validated software application (e.g., Applied BioSystems Sciex Analyst, version 1.6.2) to create a standard curve based on linear regression. The regression functions are used to calculate a best-fit line (from a set of standard concentrations in ng/mL versus peak area response) and to determine concentrations of the analyte found during sample analysis from the calculated best-fit line. For each analytical set, calibration standards are injected over the linear range of the instrument (typically 0.0250 to 2.00 ng/mL). All standards injected and their corresponding peak responses are entered into the program to create the standard curve. Weighting (1/x) is used. With no weighting, the slope of the line (curve) tends to be dominated by the highest point. When weighting of 1/concentration (1/x) is used, the slope more closely approximates the majority of the points used to construct it.

The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = detector response (peak area) for each analyte

X = analyte concentration in the sample in ng/mL

$$X = \frac{Y - \text{intercept}}{\text{Slope}} \quad \text{ng/mL}$$

The standard (calibration) curve generated for each analytical set is used for the quantitation of Pyraclonil and metabolites in the samples from the set. Correlation coefficient (r) for each calibration curve should be greater than 0.990 ($r^2$ equal to or greater than 0.98).

For the determination of Pyraclonil and metabolites in soil, soil-sediment or water (in terms of

ppb), the following equation is used:

$$Found\ (ppb) = ng/mL\ found \times \frac{Final\ Vol.\ (mL)}{Sample\ Weight\ (g)\ or\ Sample\ Vol.\ (mL)} \times DF$$

where:

| | | |
|---|---|---|
| ng/mL found | = | ng/mL of analyte found from standard curve |
| Final Vol. | = | 200 mL for soil and soil-sediment, 20 mL for water |
| DF | = | Dilution factor |
| Sample Weight | = | 20.0 g (soil and soil-sediment) |
| Sample Vol. | = | 10.0 mL (water) |

Procedural recovery data from fortified samples are calculated via the following equation:

$$Percent\ Recovery = \frac{ppb\ found - ppb\ found\ in\ control\ (average\ if\ appropriate)}{ppb\ added} \times 100$$

Example Calculations:

1. Sample Description: 89.CA.1.PD.WT.01.-0.A.25, Pyraclonil Control (water)
   (EAG ID: 85901-MV02):

$$0\ peak\ response\ (area) \rightarrow 0\ ng/mL$$

$$ppb = 0\ ng/mL\ found \times \frac{20.0\ mL}{10.0\ mL} \times 1$$

$$ppb = 0.00000$$

$$Reported\ ppb = None\ detected$$

2. Sample Description: 89.CA.1.PD.WT.01.-0.A.25+0.100 ppb
   (EAG ID: 85901-MV04),
   Pyraclonil Fortified Control @ 0.100 ppb (water):

$$37018\ peak\ response\ (area) \rightarrow 0.04803\ ng/mL$$

$$ppb = 0.04803\ ng/mL\ found \times \frac{20.0\ mL}{10.0\ mL} \times 1$$

$$ppb = 0.0961$$

$$Reported = 0.0961\ ppb$$

$$Percent\ Recovery = \frac{0.0961\ ppb - 0.000\ ppb}{0.100\ ppb} \times 100$$

$$Percent\ Recovery = 96\%$$

3. Sample Description: 89.CA.1.SL.0-2.01.BF.A.01, Pyraclonil Control (soil)
(EAG ID: 85901-MV32):

$$0\ peak\ response\ (area) \rightarrow 0\ ng/mL$$

$$ppb = 0\ ng/mL\ found \times \frac{200\ mL}{20.0\ g} \times 2$$

$$ppb = 0.000$$

$$Reported\ ppb = None\ detected$$

4. Sample Description: 89.CA.1.SL.0-2.01.BF.A.01+1.00 ppb
(EAG ID: 85901-MV34),
Pyraclonil Fortified Control @ 1.00 ppb (soil):

$$36901\ peak\ response\ (area) \rightarrow 0.04523\ ng/mL$$

$$ppb = 0.04523\ ng/mL\ found \times \frac{200\ mL}{20.0\ g} \times 2$$

$$ppb = 0.905$$

$$Reported = 0.905\ ppb$$

$$Percent\ Recovery = \frac{0.905\ ppb - 0.000ppb}{1.00\ ppb} \times 100$$

$$Percent\ Recovery = 90\%$$

## 12 STATISTICAL TREATMENT OF DATA

Statistical evaluations including percent recoveries, mean percent recoveries, standard deviations, relative standard deviations, and 95% confidence intervals were made using Microsoft Excel®. AB Sciex Analyst® software (Version 1.6.2) was used, as applicable, for the generation of standard calibration curves.

## 13 ADDITIONAL ANALYSIS

At the sponsors request and in accordance with California Department of Pesticide Regulation Environmental Monitoring Branch (CA DPR) SOP QAQC012.00 section 2.2.5 Replicate Extract Analysis, repetitive injections were performed on one California water sample extract.

### 14.3 Calculated Limits of Quantitation and Detection

The limits of quantitation (LOQ) was proposed at the initiation of the study at 1.00 ppb for soil/soil-sediment samples and 0.100 ppb for water samples. The LOD was calculated by multiplying the standard deviation of the ppb found for the LOQ fortifications for the method validation by the Student's t test value for n-1 Degrees of Freedom at the 99% confidence level for each matrix/analyte combo

| Calculated Limit of Detection (LOD) (ppb) | | | | |
|---|---|---|---|---|
| **Matrix** | **Pyraclonil** | **Amidepyraclonil** | **M-1** | **M-11** |
| California Soil | 0.0847 | 0.361 | 0.203 | 0.112 |
| California Water | 0.00974 | 0.0192 | 0.0334 | 0.0159 |
| Arkansas Soil-sediment | 0.124 | 0.0910 | 0.201 | 0.124 |
| Arkansas Water | 0.0104 | 0.036 | 0.037 | 0.008 |

### 14.5 Stability of Fortification Solutions and Calibration Standards

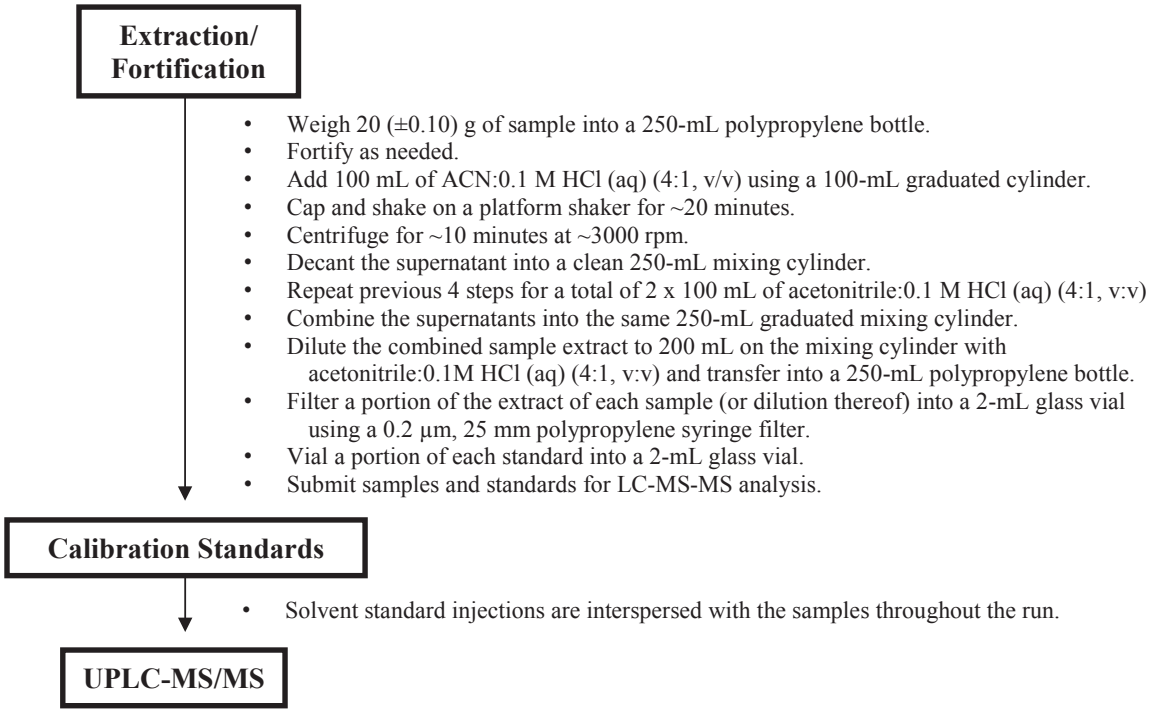
As part of this method validation study, the stability of stock standard solutions of individual analytes prepared in acetonitrile, mixed fortification standard solutions in acetonitrile, and mixed calibration standard solutions were evaluated.

The results indicate that pyraclonil, amidepyraclonil, M-1 and M-11 stock standard solutions in acetonitrile are stable for at least 95 days when stored refrigerated. Mixed fortification solutions in acetonitrile are stable for at least 69 days when stored refrigerated. Mixed calibration standards are stable for at least 69 days when stored refrigerated.

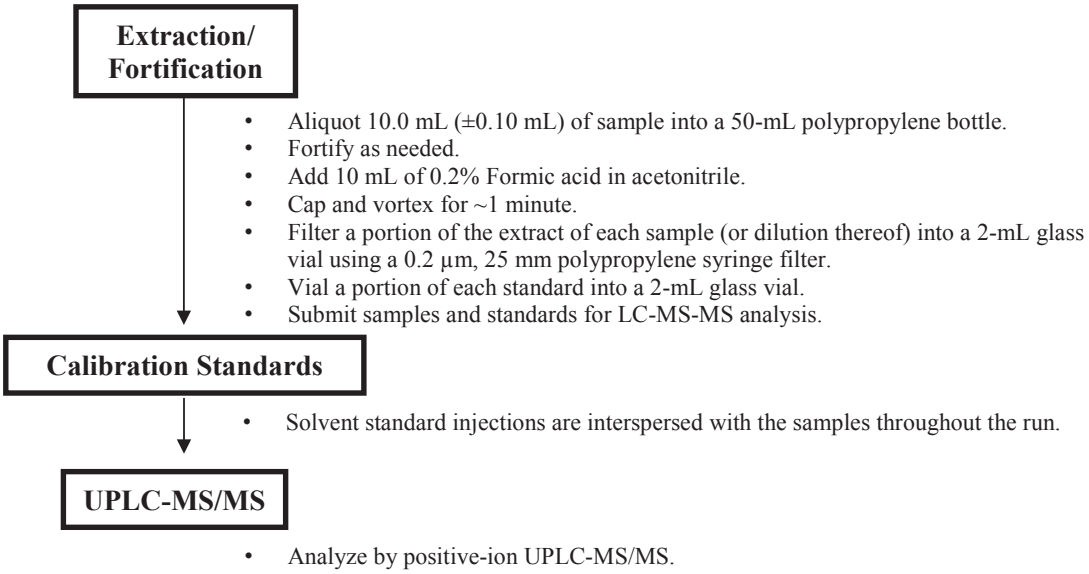
### 14.6 Protocol/SOP Deviations

Protocol Deviation No. 1, dated 28 March 2018, documented that single injections for each of the stored and freshly prepared standards were compared rather than replicate injections. As a result, no mean peak areas were calculated. It was determined that single injections for stability analysis of standards supplied adequate information for stability determination across multiple concentration ranges for all four compounds.

**Soil, Soil-sediment**



**Water**


Extraction/ Fortification
• Aliquot 10.0 mL (±0.10 mL) of sample into a 50-mL polypropylene bottle.
• Fortify as needed.
• Add 10 mL of 0.2% Formic acid in acetonitrile.
• Cap and vortex for ~1 minute.
• Filter a portion of the extract of each sample (or dilution thereof) into a 2-mL glass vial using a 0.2 µm, 25 mm polypropylene syringe filter.
• Vial a portion of each standard into a 2-mL glass vial.
• Submit samples and standards for LC-MS-MS analysis.
Calibration Standards
• Solvent standard injections are interspersed with the samples throughout the run.
UPLC-MS/MS
• Analyze by positive-ion UPLC-MS/MS.