Title

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

Data Requirements

OCSPP Guideline 850.6100

LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
°C	degrees centigrade
µg/mL	microgram per milliliter
µL	microliter
µm	micrometer
AF	Aged Fortification
aq.	aqueous
CI	Confidence Interval
FF	Fresh Fortification
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 (Std. Dev. / 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 XPE205 (Mettler Instrument Corporation, Hightstown, NJ)
Top-loading Balance (weighing samples):	Mettler ML3002E, Mettler MS12001L/03 (Mettler Instrument Corporation, Hightstown, NJ)
Centrifuge:	Sorvall Legend XTR Benchtop Centrifuge
UPLC-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 (or 20A3R) Degasser, Shimadzu CT0-20A (or CTO-20AC) 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 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 Amber vials, glass, and polypropylene tubes various sizes (for storage)
Sample Containers:	15-mL, 50-mL polypropylene centrifuge tubes 250-mL mixing cylinder 250-mL polypropylene bottles
Additional glassware/equipment:	graduated cylinder, various sizes 2-mL glass autosampler vials Sonicators, shakers, vortex mixers 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:	Optima, Fisher Scientific, Fairlawn, NJ ACS/HPLC, Honeywell Burdick & Jackson, Muskegon, MI
Formic Acid:	Optima, Fisher Scientific, Fairlawn, NJ, Acros Organic (a division of Fisher Scientific), concentration 98%+
Hydrochloric Acid (HCl):	Cert ACS, Concentrated (12.1M), Fisher Scientific, Fairlawn, NJ
Methanol:	Optima, Fisher Scientific, Fairlawn, NJ
Water:	HPLC Grade, 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 Eurofins Standard Operating Procedures. Alternate expirations may be assigned as necessary based on individual laboratory standard procedures.

4:1 Acetonitrile:0.1 M HCl

Dilute 6.61 (or 6.62 mL) mL of HCl to 800 mL with water. Combine with 3200 mL of acetonitrile. Cap and mix well. The solution is stable for 1 year when stored at room temperature.

Dilute 9.92 mL of HCl to 1200 mL with water. Combine with 4800 mL of acetonitrile. 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.

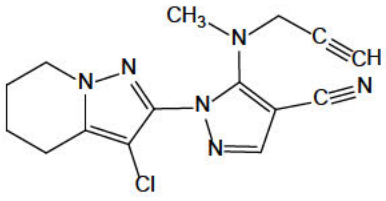
1:1:2 Acetonitrile:Methanol:Water

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

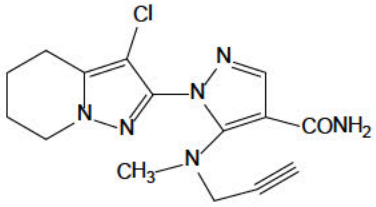
Pyraclonil:

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: 314.78 g/mol
Molecular Formula: $C_{15}H_{15}ClN_6$
Structural Formula:



Amidepyraclonil:

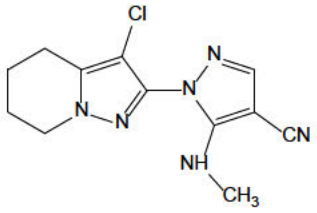
Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridin-2-yl)-5-[methyl-prop-2-ynyl)amino]-pyrazole-4-carboxamide
Molecular Weight: 332.80 g/mol
Molecular Formula: $C_{15}H_{17}ClN_6O$
Structural Formula:



M-1:

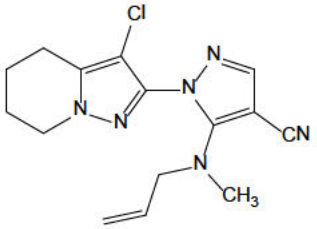
Chemical Name: 1-(3-Chloro-4,5,6,7-tetrahydropyrazolo[1,5-α]pyridin-2-yl)-5-(methylamino)-pyrazole-4-carbonitrile
Molecular Weight: 276.73 g/mol
Molecular Formula: $C_{12}H_{13}ClN_6$

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: 316.80 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-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.

<u>Calibration Solvent Standard Solutions:</u>

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 Eurofins 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 Eurofins. 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. Transferred 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. Transferred 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.

9 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.

9.1 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 DGU-20A3R Degasser, Shimadzu CT0-20A Column Oven, Shimadzu CT0-20AC 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 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 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	169	37 (quantitation)
		315	241	30 (confirmation)
		315	99	67 (confirmation)
	Amidepyraclonil	333	253	25 (quantitation)
		333	316	15 (confirmation)
		333	288	15 (confirmation)
	M-1	277	250	26 (quantitation)
		277	214	31 (confirmation)
		277	182	43 (confirmation)
		277	185	35 (confirmation)
	M-11	317	169	38 (quantitation)
		317	241	32 (confirmation)
		317	275	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	

9.2 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.

9.3 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.

10 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

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

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):

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

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

$$ppb = 0.905$$

$$Reported = 0.905\ ppb$$

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

$$Percent\ Recovery = 90\%$$

11 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.

12 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.

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To fulfill an additional California DPR SOP requirement, 31-day refrigerator storage stability was evaluated for the California water sample extracts. The results of this study indicate that residues of pyraclonil and its three metabolites (amidepyraclonil, M-1 and M-11) are stable in a CA water sample during refrigerator storage

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13.3 Calculated Limits of Quantitation and Detection

The limits of quantitation (LOQ) were 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	0.0363	0.0367	0.00770

13.4 Selectivity of Method and Confirmation of Residue Identity

The method is selective for the determination of pyraclonil and metabolites by virtue of the chromatographic separation and MS/MS detection system used. To demonstrate further confirmation, at least one additional MS/MS ion transition was monitored

13.5 Stability of Stock, Fortification, 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.

13.6 Protocol/SOP Deviations

One SOP deviation has been documented and is maintained in the study file. 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.

The deviations described above are not considered to have had a negative impact on the integrity of this study.

14 CONCLUSIONS

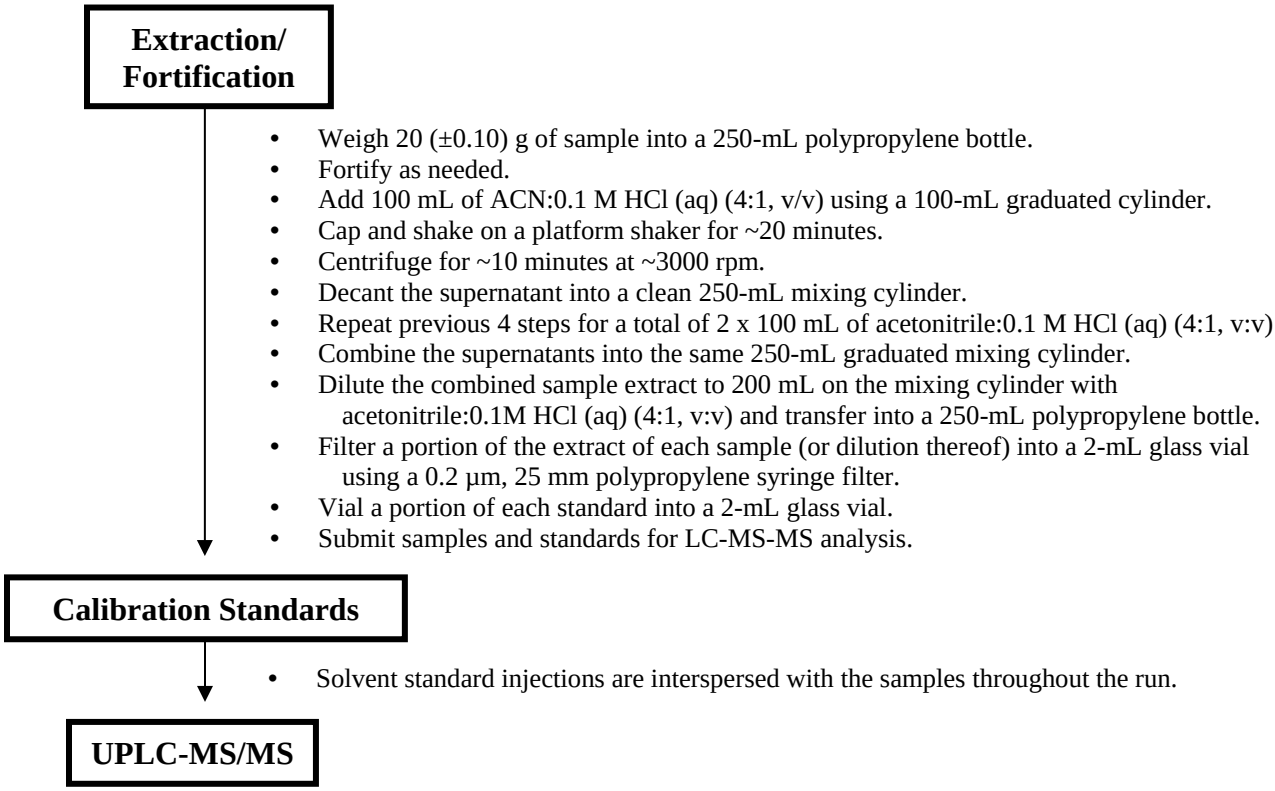
The analytical methods for the determination of pyraclonil and metabolites (amidepyraclonil, M-1 and M-11) in California and Arkansas soil/soil-sediment and water have been demonstrated to be satisfactory in terms of accuracy, precision, linearity, and selectivity. The method was validated over the concentration range of 1.00-10.0 ppb for soil/soil-sediment and 0.100-1.00 ppb for water with a calculated limit of detection individually determined for each matrix/analyte combination.

The method was selective and specific for pyraclonil and metabolites, as demonstrated by the fact that no significant matrix interference was observed in the region of elution in chromatograms of control extracts from soil, soil-sediment, or water samples in either ion transition monitored.

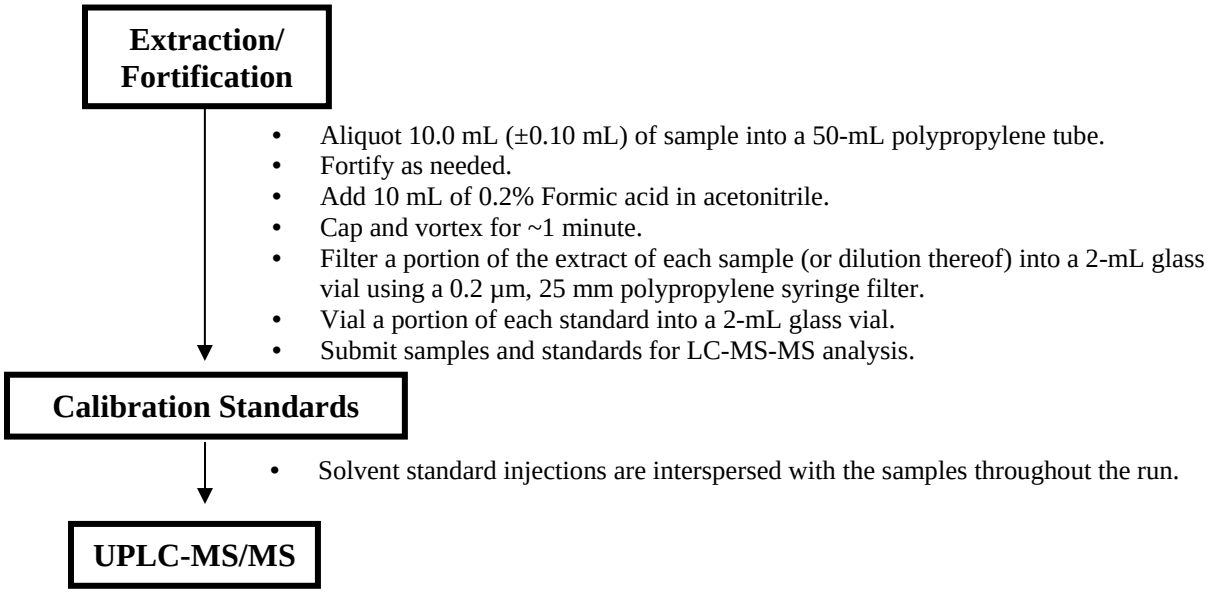
Residue confirmation was demonstrated at 1.00-10.0 ppb for soil/soil-sediment and 0.100-1.00 ppb for water based on retention time and the relative ratios of two MS/MS parent-to-fragment ion transitions detected during sample analysis.

The method meets OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation guidelines.

Soil, Soil-sediment



Water


Extraction/ Fortification
• Aliquot 10.0 mL (±0.10 mL) of sample into a 50-mL polypropylene tube.
• 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.