Title

An Analytical Method for the Determination of Residues of Pyrimethanil (AE B100309) and its Metabolite AE F132593 in Soil and Water Using LC/MS/MS

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1.0 SUMMARY

An analytical method was developed to determine the residues of pyrimethanil and AE F132593 in soil at a target LOQ of 5 ng/g and in water at a target LOQ of 0.5 ng/g.

Residues of pyrimethanil and AE F132593 are extracted from a 10-g soil sample by Soxhlet extraction with 9:1 (v/v) acetonitrile/water for 6 hours.[1] An isotopic internal standard of pyrimethanil is added to the extract. An aliquot of the extract is evaporated and reconstituted for analysis by liquid chromatography/tandem mass spectroscopy (LC/MS-MS).

For water samples, a 10-g aliquot is amended with an isotopic internal standard. The resultant sample is analyzed by LC/MS-MS.

2.0 BACKGROUND

Pyrimethanil (the active ingredient of SCALA), a fungicide, is registered for use on almonds, pistachios, bulb vegetables, grapes, lemons, stone and pome fruits, potatoes, strawberries, and tomatoes. The analytical method presented in this report is designed to measure residues of pyrimethanil and the metabolite AE F132593 (2-Amino-4,6-dimethylpyrimidine) in soil and water in support of continued registration.

3.0 APPARATUS

(Functional equivalents may be substituted)

- Various general laboratory glassware and utensils
- Soxhlet apparatus with a 250-mL boiling flask and condenser
- MicroMan pipettors and tips (M250, M50, and M1000)
- Rotary evaporator (Buchi)
- Phenomenex HPLC column, Kinetix XB-C18, 100mm x 2.1mm, 2.6 µm (Part No: 00D-4496-AN)
- Vortex mixer
- LC/MS-MS triple quadrupole instrument equipped with electrospray ionization (ESI) interface, HPLC pumps, column oven, and an autosampler

4.0 REAGENTS AND CONSUMABLES

(Functional equivalents may be substituted)

- Acetonitrile (ACN; Optima Grade, Fisher Part No. A996-4)
- Ammonium formate (Fisher Part No. A115-50)
- Formic Acid 99% (Acros, Part No. 14793-0010)
- Methanol (MeOH; Optima Grade; Fisher Part No. A456-4)
- Water (Optima Grade; Fisher Part No. W7-4)
- 9:1 (v/v) ACN/water. Combine 900 mL of ACN with 100 mL of water. Mix well.
- Soxhlet extraction thimbles to fit the extraction apparatus.
- Glass wool
- Disposable centrifuge tube (15-mL polypropylene)
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Glass culture Tube 20x150mm (Fisher Part No. 14-961-33)
- 1:9 (v/v) MeOH/water with 10 *mM* ammonium formate and 0.12 mL/L of formic acid. Add 0.63 g of ammonium formate to 900 mL of water. Add 0.12 mL of formic acid. Mix until the ammonium formate is dissolved. Add 100 mL of MeOH and mix well.
- 9:1 (v/v) MeOH/water with 10 *mM* ammonium formate and 0.12 mL/L of formic acid. Add 0.63 g of ammonium formate to 100 mL of water. Add 0.12 mL of formic acid. Mix until the ammonium formate is dissolved. Add 900 mL of MeOH and mix well.

5.0 PREPARATION OF STANDARD SOLUTIONS

The pyrimethanil and AE F132593 analytical standards and the isotopic internal standard (IS) pyrimethanil-$^{13}C,^{15}N_2$ are required. The pyrimethanil IS is used for both AE F132593 and pyrimethanil. These standards may be obtained from Bayer CropScience, 2 T.W. Alexander Drive, Research Triangle Park, North Carolina, 27709. Additional details about these chemicals are given in Appendix 1.

The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.

Note: The following procedure is an example description of how these standard solutions may be prepared. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Volumetric glassware and calibrated pipets should be used in the preparation of all analytical standards. Corrections for standard purities should be applied when expressing standard concentrations.

5.1 Primary Standards

Reference standards and internal standard primary solutions are prepared as shown below.

Reference Standard	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Pyrimethanil	~10	50.0	ACN	~200
AE F132593	~10	50.0	ACN	~200
Pyrimethanil-$^{13}C,^{15}N_2$ IS	~5	50.0	ACN	~100

Note: Primary solutions are prepared in **native analyte equivalents.** The concentration of the primary solutions should also be corrected for purity of the standard during the initial preparation. Primary solutions should be stored in a freezer when not in use.

5.2 Secondary Standards

Mixed secondary reference and internal standard solutions are prepared from the primary solutions as shown below. Take the appropriate aliquot of each of the stock solutions to give the required mixed secondary standard concentration.

Compound	Primary Standard Concentration (µg/mL)	Aliquot (mL)	Final Volume (mL)	Mixed Secondary Standard Concentration (µg/mL)	Solvent
Pyrimethanil	~200	~12.5	50.0	50.00	ACN
AE F132593	~200	~12.5			
Pyrimethanil-$^{13}C,^{15}N_2$ IS	~100	~2.5	50.0	5.000	ACN

Additional secondary reference standard solutions are prepared as shown below.

Concentration of Mixed Native Standard Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Concentration of Mixed Native Secondary Standard Solution (µg/mL)	Solvent
50.00	5.00	50.0	5.000	ACN
5.000	5.00	50.0	0.500	ACN
0.500	5.00	50.0	0.050	ACN

Secondary standards should be stored in a refrigerator when not in use.

5.3 Calibration Standards

Note: Additional or alternate calibration standards may be prepared when necessary; however, the concentration of IS must remain the same in all calibration standards. Calibration solutions are diluted to volume with water and should contain <5% organic solvent, preferably <2% organic solvent.

Concentration of Native Standard Solution used for dilution (µg/mL)	Aliquot of Native Standard Solution Taken (mL)	Concentration of IS Solution used for dilution (µg/mL)	Aliquot IS Taken (mL)	Dilution Volume (mL)	Concentration of Native in Calibration Solution (ppb)	Concentration of IS in Calibration Solution (ppb)
50.00	0.750	5.000	0.250	25	1500	50
50.00	0.250	5.000	0.250	25	500	50
50.00	0.075	5.000	0.250	25	150	50
5.000	0.250	5.000	0.250	25	50	50
5.000	0.075	5.000	0.250	25	15	50
0.500	0.250	5.000	0.250	25	5.0	50
0.500	0.075	5.000	0.250	25	1.5	50
0.050	0.250	5.000	0.250	25	0.5	50
0.050	0.125	5.000	0.250	25	0.25	50

Calibration standards should be stored in a refrigerator when not in use.

6.0 EXTRACTION PROCEDURE

6.1 Water Sample Preparation

1. Weigh 10 $\pm$ 0.10 grams of water into a 20x150mm glass culture tube.

2. If necessary, fortify the recovery samples with the appropriate standard solution. Vortex the sample to mix well.

3. Add 0.100 mL of the 5.000 µg/mL IS solution to the sample in the culture tube. Vortex the sample to mix well.

4. Transfer an aliquot of the sample into an HPLC vial for LC/MS-MS analysis. Analyze against a linearity curve running from 0.25 ppb to 50 ppb.

6.2 Soil Sample Extraction

1. Weigh 10 ± 0.10 grams of soil into a Soxhlet extraction thimble.

2. If necessary, fortify the recovery samples with the appropriate standard solution. Allow the fortified sample to sit for a minimum of 10 minutes.

3. Lightly plug the top of the Soxhlet extraction thimble with glass wool.

4. Assemble a 250-mL boiling flask containing 125-mL of 9:1 v/v ACN:water, followed by a Soxhlet extractor containing the extraction thimble with the sample, followed by a condenser.

5. Place a heat source on/under the boiling flask to reflux the solvent and to cause the Soxhlet apparatus to cycle. Allow the apparatus to cycle for 6 hours.

6. Cool the apparatus and rinse the condenser with about 10-20 mL of ACN and allow the rinse to collect in the Soxhlet extractor. Disassemble the Soxhlet apparatus and decant any residual solvent from the apparatus into the 250-mL boiling flask. Discard the extraction thimble containing the soil.

7. Add 0.100 mL of the 5.000 µg/mL IS solution to the extract in the 250-mL boiling flask and swirl to mix. Allow the solid material in the flask to settle.

8. Transfer a 12-15 mL aliquot of the sample supernatent to a 20x150mm culture tube. Evaporate the sample to near dryness on a TurboVap (bath temperature 45-50℃).

5. Add 1.0-mL of water to the culture tube to redissolve the sample (sonicate if necessary). Transfer the sample into an HPLC vial for LC/MS-MS analysis. Analyze against a linearity curve running from 1.5 ppb to 1500 ppb.

7.0 ANALYSIS BY LC/MS/MS

7.1 Analytical Procedure

Step 1. Using the recommended procedures listed below; analyze the calibration standard solutions (if necessary, additional standard solutions may be added).

Step 2. Analyze an aliquot of each of the analytical samples.

Note: Up to 20 sample analyses can be made after the analysis of the standard solutions.

Step 3. Repeat Step 1.

Step 4. When necessary analyze additional samples and standard solutions. Always finish the procedure with a set of calibration solutions.

7.2 HPLC Conditions

Note: The analyst should optimize chromatographic conditions to obtain satisfactory chromatography. As the HPLC column ages, the retention times of the analytes may change.

Mobile Phase A: 1:9 (v/v) MeOH/water with 10 *mM* ammonium formate and 0.12 mL/L of formic acid
Mobile Phase B: 9:1 (v/v) MeOH/water with 10 *mM* ammonium formate and 0.12 mL/L of formic acid
Column Oven: 50º C
HPLC column: Phenomenex Kinetix XB-C18, 100mm x 2.1mm, 2.6 µm
Injection volume: 10 µL (adjust as needed)
HPLC Gradient Conditions:

Time (min)	Mobile Phase B%	Flow rate µL/min
0.00	30	250
0.30	30	250
0.31	95	250
2.50	95	250
2.51	30	250
5.00	30	250

Approximate Retention Times:

Analyte	Retention Time (min)
AE F132593	1.5
Pyrimethanil	2.8

7.3 Mass Spectrometer Conditions

The triple quadrupole MS/MS instrument is operated in the Selected Reaction Monitoring or Multiple Reaction Monitoring mode (SRM/MRM). Parent analyte ions are selected and subjected to collision-induced dissociation to create product ions.

The collection of data for two product ions per analyte is recommended; one primary product ion serving for quantitation and a second to confirm any detected residues (if necessary). The product ions selected by the development laboratory are given in the table below. Alternate product ions may be selected, if necessary, provided the analytical method is validated under those conditions.

Ionization Source: API interface-electrospray

Analyte Name	Polarity	Q1 Mass (m/z), Parent ion	Q3 Mass (m/z), Primary ion	Q3 Mass (m/z), Confirmatory ion
AE F132593	Positive	124.20	107.05 (CE=19)	82.2 (CE=22)
Pyrimethanil	Positive	200.15	107.05 (CE=24)	182.1 (CE=29)
Pyrimethanil-$^{13}C,^{15}N_2$ IS	Positive	203.15	110.05 (CE=24)	--

Note: The analyst must optimize the instrument conditions to obtain satisfactory system response prior to use. This includes confirmation of the HPLC conditions and retention times, as well as, confirmation of MS/MS sensitivity, linearity, and product ion selection.

8.0 CALCULATION OF RESULTS

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Note: The pyrimethanil IS is used as an internal standard in the calculations for both AE F132593 and pyrimethanil.

Residue concentrations were determined using calibration curves generated after each analysis using the LC/MS-MS software and fitting the data to a linear regression with 1/X weighting.

$$Y = MX + Bm$$

where: **X** is the concentration of the reference standard in ppb
M is the calibration line slope
B is the calibration line Y-intercept
Y is the native peak area/IS peak area ratio

The residue levels of the samples were determined using the LC/MS-MS software with the following equation:

$$Rem\quad e\,fomn\quad (ppb) = \frac{(Y\text{-}B)m}{Mm}$$

Residue levels beyond the calibration curve: In some cases, an unknown sample contains residues at a level above the calibration curve. If so, the preferred strategy is to extend the calibration curve to cover the apparent residue level in the unknown sample. If this is not an option, contact the development laboratory for instructions on how to proceed.

8.1 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility. Fortification experiments are intended to be used for data collection methods or establishing and validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$Recovery\ (\%) = \frac{Meam\ remRem\quad em}{Fort\ f\ e\quad Amo\quad ntm} \times 100m$$

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ or other appropriate level with fortification solutions.

9.0 NOTES ON THE ANALYSIS

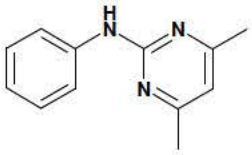
The chromatographic system employs a step gradient. The AE F132593 metabolite elutes first under the isocratic conditions of 30% solvent B. Then the chromatographic solvent gradient is 'stepped' suddenly to 95% solvent B to quickly elute pyrimethanil. The HPLC pump used for method development has a 1.0 min dead volume at a flow rate of 0.25 mL/min (there is a 1.0 min lag between a solvent composition change at the pump and the solvent composition changing at the HPLC column). The solvent gradient step is timed to occur as the AE F132593 metabolite elutes (1.0 min pump dead volume + 0.2 min column dead volume + 0.3 min step time = 1.5 min elution time for AE F132593). If an HPLC pump with a smaller dead volume is used, the gradient step time should be increased accordingly.

The starting isocratic solvent mixture contains about 30% organic solvent. However, the analysis of samples and quantitation standards containing less than 5% organic solvent show considerably improved AE F132593 chromatographic peak shape. Use of a 10-µL injection loop also improved the AE F132593 chromatographic peak shape.

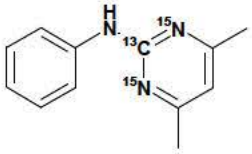
The range of standard concentrations listed in Section 5.3 Calibration Standards was selected to cover residues seen in prior soil dissipation studies. The development laboratory observed linear instrument response for pyrimethanil across the range of 0.25 ppb to 1500 ppb. The laboratory observed linear instrument response for the AE F132593 metabolite from 0.25 ppb to 150 ppb.

Appendix 1 Test and Reference Substances

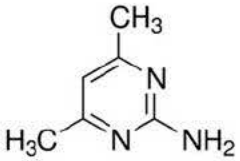
The toxicities of these chemicals have not been precisely determined. Thus, each chemical must be treated as a potential health hazard. Exposure to these chemicals should be reduced to the lowest reasonable level.



Code Name: Pyrimethanil (AE B100309)
Molecular Formula: $C_{12}H_{13}N_3$
Molecular Weight: 199.25 g/mol



Code Name: Pyrimethanil-^{13}C-$^{15}N_2$
Molecular Formula: $C_{11}{}^{13}CH_{13}N^{15}N_2$
Molecular Weight: 202.23 g/mol



Code Name: AE F132593
Molecular Formula: $C_6H_9N_3$
Molecular Weight: 123.16 g/mol