Appendix 6 Analytical Method HE-001-W17-01

Title

An Analytical Method for the Determination of Residues of Fluoxastrobin (HEC-5725) and its Metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in Water Using LC/MS/MS

-Analytical Method-

Test Substance

Fluoxastrobin
(HEC-5725)

Guideline Number

US EPA Test Guideline OCSPP 860.1340: Residue Analytical Method

OECD Guidance Document on Pesticide Residue Analytical Methods, Series on Testing and Assessment Document 72 and Series on Pesticides: Document 39, August 2007 (OECD Guideline, ENV/JM/MONO (2007) 17, Aug 13, 2007)

PMRA Residue Chemistry Guidelines, Regulatory Directive 98-02, Section 3, Residue Analytical Method, June 1998

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

Bayer Method HE-001-W17-01

An Analytical Method for the Determination of Residues of Fluoxastrobin (HEC-5725) and its Metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in Water Using LC/MS/MS

1.0 SUMMARY

An analytical method was developed to determine the residues of fluoxastrobin (HEC-5725) and its metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in water.

Residues of fluoxastrobin and its metabolites in water are spiked with isotopic internal standards. A sample aliquot is analyzed for fluoxastrobin (HEC-5725), HEC 5725-deschlorophenyl and HEC 5725-oxazepine by LC/MS/MS. Quantification is based on a comparison of peak areas with those of known standards.

This method was developed to analyze residues of fluoxastrobin and its metabolites in water at a target limit of quantification (LOQ) of 0.05 ng/g, but can be adjusted as required.

2.0 BACKGROUND

. The analytical method presented in this report is designed to measure residues of fluoxastrobin (HEC-5725), HEC 5725-deschlorophenyl and HEC 5725-oxazepine in water using isotopically-labeled internal standards and LC/MS/MS detection.

3.0 APPARATUS

Functional equivalents may be substituted.

- Various general laboratory glassware and utensils.
- MicroMan pipettors and tips (M250, M50, and M1000).
- Eppendorf 5810 Centrifuge
- Phenomenex Kinetex C18 100 mm x 2.1 mm 1.7 µm particle size (Part No: 00D-4498-AN)
- Thermo TSQ Quantiva chromatograph/mass spectrometer (LC-MS/MS) equipped with electrospray ionization (ESI) interface, Thermo Vanquish UHPLC pumps, Thermo Vanquish column compartment and a Thermo Vanquish autosampler, and LCQuan/Xcalibur software

4.0 REAGENTS AND CONSUMABLES

Functional equivalents may be substituted

- Methanol (Optima Grade, Fisher Part No. A456-4)
- Formic acid 99% (Acros, Part no. 14793-0010)
- Water (Optima Grade; Fisher Part No. W7-4)

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

- Acetic acid glacial (Fisher Part No. A38-500)
- 800/200/0.1 (v/v/v) water/acetonitrile/acetic acid. Combine 800 mL water, 200 mL acetonitrile, and 1 mL acetic acid. Mix well.
- 0.1% formic acid in methanol. Add 1 mL formic acid to 1000 mL methanol. Mix well.
- 0.1% formic acid in water. Add 1 mL formic acid to 1000 mL water. Mix well.
- HPLC vials and caps (2-mL, National Scientific, Part Nos. C4011-5W and C4011-55)
- Disposable Glass pipet, 5 mL (Fisher Part No. 13-678-31H)
- 60 mL Clear Vials Boro with Septa (Thermo Part No. S236-0060)
- Culture Tube 20 x150mm (Fisher Part No. 14-961-33)
- Kimble™ Volumetric flasks 25 mL and 50 mL (Fisher Part No. S00122)

5.0 PREPARATION OF STANDARD SOLUTIONS

The native reference standards used in this method are: fluoxastrobin (HEC-5725), HEC 5725-deschlorophenyl and HEC 5725-oxazepine. The isotopic internal standards (IS) standard required for this method are: HEC 5725-dioxazin-D_4, and HEC 5725-deschlorophenyl-dioxazin-D_4. 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 Standard Solutions

Primary native (reference) standard solutions (Table 1) and primary internal standard solutions (Table 2) are prepared as shown below.

Table 1. Primary reference (native) standard solution preparation

Native Reference Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
Fluoxastrobin (HEC-5725)	~5	50.0	ACN	~100
HEC 5725-deschlorophenyl	~5	50.0	ACN	~100
HEC 5725-oxazepine	~2.5	50.0	ACN	~50

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

Table 2. Primary internal standard solution preparation.

Isotopic Internal Standards	Weight (mg)	Volume (mL)	Solvent	Final Concentration (µg/mL)
HEC 5725-dioxazin-D_4	~5	50.0	ACN	~100
HEC 5725-deschlorophenyl-dioxazin-D_4	~5	50.0	ACN	~100

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 Standard Solutions

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

Table 3. Secondary mixed reference standard solution preparation.

Compound	Primary Standard Concen-tration (µg/mL)	Aliquot from Primary Standard (mL)	Final Volume (mL)	Mixed Secondary Standard Final Concentration (µg/mL)	Solvent
Fluoxastrobin (HEC-5725)	~100	~0.5	50.0	1.0	ACN
HEC 5725-deschlorophenyl	~100	~0.5			
HEC 5725-oxazepine	~50	~1.0			
HEC 5725-dioxazin-D_4	~100	~0.1	50.0	0.2	ACN
HEC 5725-deschlorophenyl-dioxazin-D_4	~100	~0.1			

Additional secondary reference standard solutions are prepared as shown below.

Table 4. Additional secondary mixed native secondary standard solution preparation.

Concentration of Mixed Native Standard Solution used for dilution (µg/mL)	Aliquot Taken (mL)	Dilution Volume (mL)	Final Concentration of Mixed Native Secondary Standard Solution (µg/mL)	Solvent
1.0	1.0	50.0	0.02	ACN

All secondary standards should be stored in a freezer when not in use.

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

5.3 Calibration Standards

Note: Additional standards may be prepared when necessary; however, the concentration of internal standard must remain the same in all calibration standards. Calibration solutions are diluted to volume in 800/200/0.1 (v/v/v) water/acetonitrile/acetic acid.

Table 5. Calibration standard solutions

Concentration of Native Standard Solution used for dilution (ppm)	Concentration of Internal Standard Solution used for dilution (ppm)	Aliquot Native Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Native in Calibration Solution (ppb)	Concentration of IS in Calibration Solution (ppb)
0.02	0.2	0.075	0.0625	50.0	0.03	0.25
0.02	0.2	0.15	0.0625	50.0	0.06	0.25
0.02	0.2	0.3	0.0625	50.0	0.12	0.25
0.02	0.2	0.625	0.0625	50.0	0.25	0.25
0.02	0.2	1.25	0.0625	50.0	0.5	0.25
0.02	0.2	2.5	0.0625	50.0	1	0.25

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

6.0 EXTRACTION PROCEDURE

6.1 Sample Extraction

Fortified recovery samples are prepared by spiking a known level of the appropriate standard(s) into a sample. A control sample should also be prepared, using the same samples as the fortified sample, to determine the residue levels, real or apparent, found in this sample and the % recovery calculated as described in Section 8.1.

1. Weigh 50 ± 0.05 g of water into a suitable stoppered container.
2. Fortify the recovery samples at the desired fortification level with the appropriate standard solution.
3. Add 0.0625 mL of the 0.2 µg/mL internal standard and mix well
4. Analyze a sample aliquot by LC/MS/MS

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

7.0 ANALYSIS BY LC/MS/MS

7.1 Analytical Procedure

Step 1. Using the recommended procedures listed below; analyze a minimum of six 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 calibration 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: 0.1% Aqueous Formic Acid
Mobile Phase B: 0.1% Methanol Formic Acid
Oven: 40 ºC
Phenomenex Kinetex C18 100 mm x 2.1 mm 1.7 µm particle size
Injection Volume: 50 µL (adjust as needed)

Time (min)	Mobile Phase B%	Flow rate µL/min
0.00	5	300
0.10	5	300
6.00	95	300
7.00	95	300
8.00	5	300
11.00	5	300

Analyte	Approx Retention Time (min)
Fluoxastrobin (HEC-5725)	6.2
HEC 5725-deschlorophenyl	4.2
HEC 5725-oxazepine	6.4

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

7.3 Mass Spectrometer Conditions

The MS/MS instrument is operated in the multiple reaction monitoring mode (MRM). Precursor ions are selected and product ions created by collision-induced dissociation.

Two product ions per analyte are listed in Table 7, one product ion (MRM-transition) serving for quantitation and the second ion to be used, only if required, to confirm the presence of any detected residues.

Note: The following recommended conditions were used on a Thermo TSQ Quantiva instrument but the analyst should optimize the mass spectrometer conditions to obtain satisfactory system response prior to use.

Ionization Mode:	API interface-electrospray ionization mode
Sheath/Aux/Ion Sweep Gas	Nitrogen
Collision Gas	Argon
Spray Voltage (V)	3700
Vaporizer Temp (°C):	400
Sheath Gas Pressure	50
Aux Gas Pressure	15
Ion Sweep Gas Pressure	2
Ion Transfer Tube Temp (°C)	350
Q2 Collision Gas (mT):	1.5

Table 6. MS/MS Parameters for the determination of Fluoxastrobin (HEC-5725) and its Metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine

Analyte Name	Polarity	Q1 Mass (amu)	Q3 Mass (amu)	Collision Energy (V)	Resolution for Q1MS (amu)	Resolution for Q3MS (amu)
Fluoxastrobin (HEC-5725)	Pos	459.1	188	40	0.7	0.7
Fluoxastrobin (HEC-5725) IS	Pos	463.1	188	40	0.7	0.7
HEC 5725-deschlorophenyl	Pos	349.1	102.1	30	0.7	0.7
HEC 5725-deschlorophenyl IS	Pos	353.1	102.1	30	0.7	0.7
HEC 5725-oxazepine	Pos	409.1	169.1	30	0.7	0.7

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

Table 6 (Continued)

Fluoxastrobin (HEC-5725) Confirmatory	Pos	459.1	427	20	0.7	0.7
Fluoxastrobin (HEC-5725) IS Confirmatory	Pos	463.1	431	20	0.7	0.7
HEC 5725-deschlorophenyl Confirmatory	Pos	349.1	317.1	20	0.7	0.7
HEC 5725-deschlorophenyl IS Confirmatory	Pos	353.1	321.1	20	0.7	0.7
HEC 5725-oxazepine Confirmatory	Pos	409.1	365	20	0.7	0.7

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.

Residue concentrations were determined using calibration curves which were generated after each analysis using LCQuan or Xcalibur software using linear regression with 1/x weighting.

The standards were fit to the linear equation:

$$Y = MX + B \text{ with } 1/x \text{ weighting.}$$

Where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

After regression coefficients were calculated, the residue in ng/g was determined using the following equation,

$$\text{Residue (ng/g)} = \frac{(Y-B)}{M}$$

For HEC 5725-oxazepine whose internal standard is not available, HEC 5725-dioxazin-d4, the internal standard for the parent fluoxastrobin (Q1/Q3=463.1/188), may serve as a surrogate.

Analyst software was used to calculate the amount of fluoxastrobin (HEC-5725) and its metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in ng/g for each sample and the percent recovery for the fortified samples.

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

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

calibration curve to cover the unknown sample, if this is not an option contact the development laboratory for instructions on how to proceed

Recovery Experiments

Recovery (fortification) experiments may be performed as needed to monitor method efficiency and reproducibility. Recovery experiments are intended to be used for data collection methods or establishing and validating method efficiency and are prepared by adding a known amount of native standard solution to a sample aliquot and preparing the sample for analysis as described in Section 6.

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

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

Where: R = ng/g of target analyte found in fortified (recovery) sample
S = ng/g of target analyte found in control sample, real or apparent
T = theoretical ng/g in fortified sample

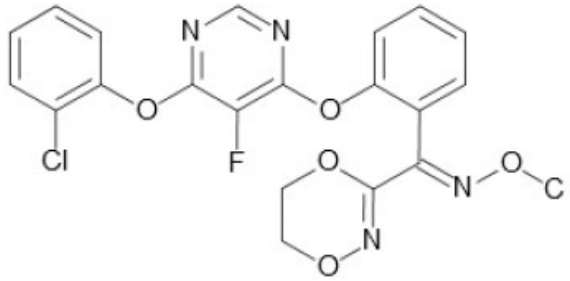
Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Recovery samples are fortified prior to extraction at the LOQ of 0.05 ng/g with fortification solutions. Calculate the final residue for the control (S) and fortified control (R) samples.

APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

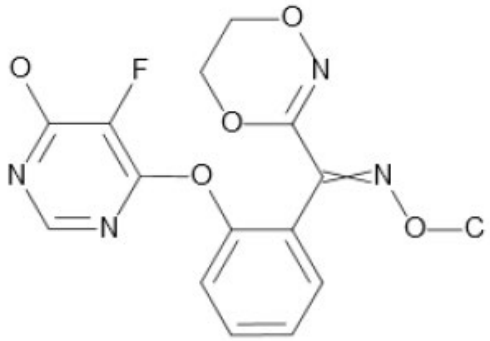
Appendix 1 Reference Substances

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:	Fluoxastrobin (HEC-5725)
CAS Name:	
CAS Number:	361377-29-9
Molecular Formula:	$C_{21}H_{16}ClFN_4O_5$
Molecular Weight:	458.83 g/mol
Chemical Structure:	



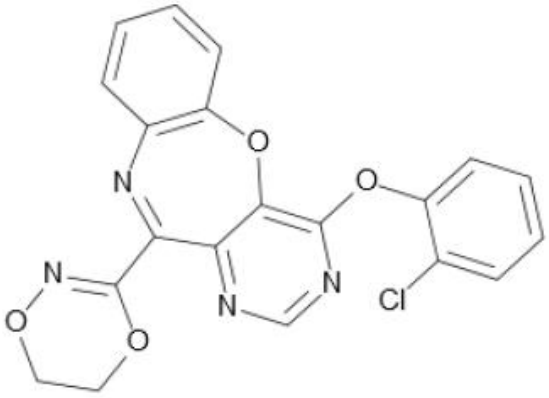
Code Name:	HEC 5725-deschlorophenyl
CAS Number:	Unavailable
Molecular Formula:	$C_{15}H_{13}FN_4O_5$
Molecular Weight:	348.29 g/mol
Chemical Structure:	



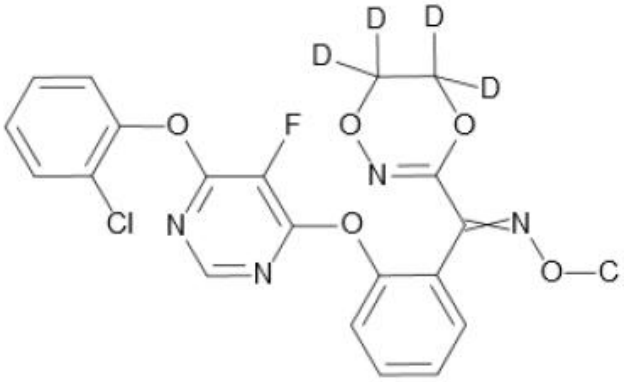
APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

Appendix 1 Continued

Code Name: HEC 5725-oxazepine
CAS Number: Unavailable
Molecular Formula: $C_{20}H_{13}ClN_4O_4$
Molecular Weight: 408.79 g/mol
Chemical Structure:



Code Name: HEC 5725-dioxazin-d4
CAS Number: Unavailable
Molecular Formula: $C_{21}H_{12}ClD_4FN_4O_5$
Molecular Weight: 462.85 g/mol
Chemical Structure:



APPENDIX 6 ANALYTICAL METHOD HE-001-W17-01, continued

Appendix 1 Continued

Code Name:	HEC 5725-deschlorophenyl-dioxazin-d4 (E)
CAS Number:	Unavailable
Molecular Formula:	$C_{15}H_9D_4FN_4O_5$
Molecular Weight:	352.31 g/mol
Chemical Structure: