TITLE

Independent Laboratory Validation of 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

- Final Report -

Test Items

Fluoxastrobin (HEC-5725)
HEC 5725-deschlorophenyl
HEC 5725-oxazepine

Data Requirement

US EPA Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (OPPTS 850.7100) and OPPTS (OCSPP) 860.1340(c)(6)]

INTRODUCTION

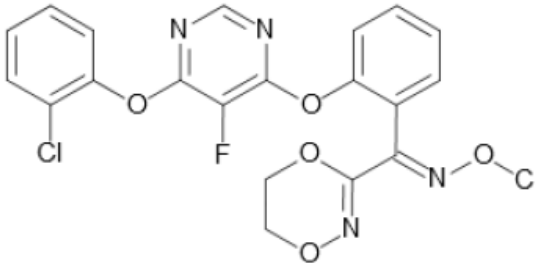
The purpose of this study was to validate the analytical method HE-001-W17-01 for fluoxastrobin (HEC-5725) and its metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in water. This Independent Laboratory Validation (ILV) was conducted under US EPA Test Guidelines OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation (OPPTS 850.7100) and OPPTS (OCSPP) 860.1340(c)(6)]. This study was conducted in compliance with the EPA FIFRA Good Laboratory Practice Standards, 40 CFR Part 160.

3. MATERIALS AND METHODS

3.1. TEST ITEMS/REFERENCE SUBSTANCES

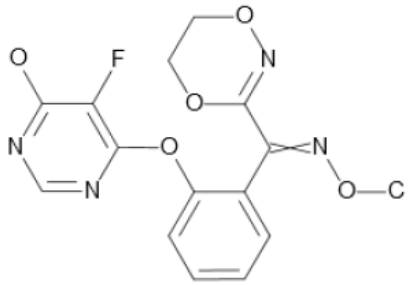
Only sufficiently characterized and certified substances were used as reference items.

Fluoxastrobin (HEC-5725):



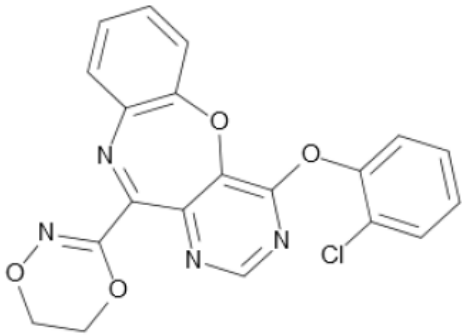
CAS Name:	(1E)-[2-[[6-(2-Chlorophenoxy)-5-fluoro-4-pyrimidinyl]oxy]phenyl](5,6-dihydro-1,4,2-dioxazin-3-yl)methanone O-methyloxime
CAS Number	361377-29-9
Molecular Formula:	$C_{21}H_{16}ClFN_4O_5$
Molecular Weight:	458.83 g/mol
ID No.:	K-2237
Purity:	99.2%
Expiration Date:	3/21/23
Date Of Analysis:	3/21/13
Storage Conditions:	Freezer
Source:	Bayer CropScience, Research Triangle Park

Standard Name: **HEC 5725-deschlorophenyl**



CAS Name:	6-[2-[(5,6-Dihydro-1,4,2-dioxazin-3-yl)(methoxyimino)methyl]phenoxy]-5-fluoro-4(1*H*)-pyrimidinone
CAS Number:	Unavailable
Molecular Formula:	$C_{15}H_{13}FN_4O_5$
Molecular Weight:	348.29 g/mol
ID No.:	K-2242
Purity:	99.2%
Expiration Date:	3/4/18
Date Of Analysis:	3/4/14
Storage Conditions:	Freezer
Source:	Bayer CropScience, Research Triangle Park

Standard Name: **HEC 5725-oxazepine**

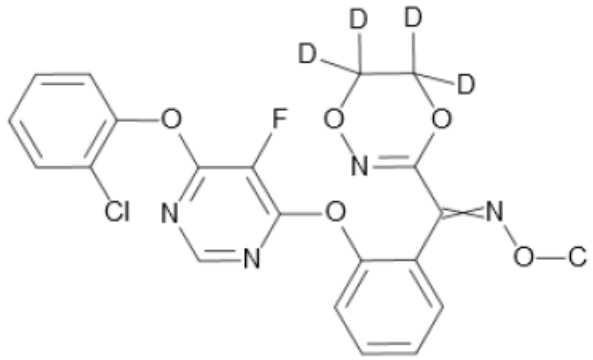


CAS Name:	4-(2-Chlorophenyl)-11-(5,6-dihydro-1,4,2-dioxazin-3-yl)pyrimido[5,4-*b*]benzoxazepine
CAS Number	Unavailable
Molecular Formula:	$C_{20}H_{13}ClN_4O_4$
Molecular Weight:	408.79 g/mol
ID No.:	K-1074
Purity:	98.8%
Expiration Date:	1/3/27
Date Of Analysis:	1/3/17
Storage Conditions:	Freezer
Source:	Bayer CropScience, Research Triangle Park

3.2.

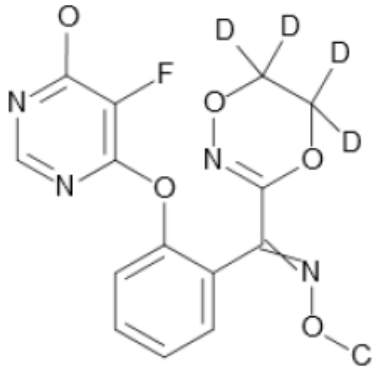
INTERNAL STANDARD

Standard Name: **HEC 5725-dioxazin-d4**



CAS Name: [2-[[6-(2-Chlorophenoxy)-5-fluoro-4-pyrimidinyl]oxy]phenyl](5,6-dihydro-d_2-1,4,2-dioxazin-3-yl-5,6-d_2)methanone-O-methyloxime
CAS Number: Unavailable
Molecular Formula: $C_{21}H_{12}ClD_4FN_4O_5$
Molecular Weight: 462.85 g/mol
ID No.: K-902
Purity: 98.0%
Expiration Date: 1/3/27
Date Of Analysis: 1/3/17
Storage Conditions: Freezer
Source: Bayer CropScience, Research Triangle Park

Standard Name: **HEC 5725-deschlorophenyl-dioxazin-d4 (E)**



CAS Name: (5,6-Dihydro-d_2-1,4,2-dioxazin-3-yl-5,6-d_2)[2-[(5-fluoro-6-hydroxy-4-pyrimidinyl)oxy]phenyl]methanone *O*-methyloxime
CAS Number: Unavailable
Molecular Formula: $C_{15}H_9D_4FN_4O_5$
Molecular Weight: 352.31 g/mol
ID No.: K-909
Purity: 97.9%
Expiration Date: 1/3/27
Date Of Analysis: 1/3/17
Storage Conditions: Freezer
Source: Bayer CropScience, Research Triangle Park

Characterization data for the reference substance (analytical standards) and internal standard are maintained by Bayer CropScience.

The test/reference substance (analytical standard) and internal standard used in this study were procured from Bayer CropScience and stored as directed on “Analytical Standards Chain of Custody” and “Certificate of Analysis” documents. All solutions made from the reference substance (analytical standard) were stored according to the method.

3.3. TEST SYSTEMS

The analytical method HE-001-W17-01 was evaluated in water from the United States. Sample Chain of Custody (CoC) and analytical results are supplied in the raw data. The water was collected on September 15, 2017 from the Ecotoxicology unit at SynTech Research Laboratory Services, Inc. in the state of Kansas (KS).

3.4. SAMPLE PREPARATION AND EXTRACTION

Bayer Analytical Method HE-001-W17-01 was used for the analysis of fluoxastrobin (HEC-5725) and its metabolites HEC 5725-deschlorophenyl and HEC 5725-oxazepine in water. The preparation of the sample residues were as follows:

Water samples (50 g) were weighed into polypropylene 50 mL centrifuge tubes, fortified, internal standard was added, then capped and mixed well. An aliquot of the supernatant was then removed and then analyzed by LC/MS/MS. Possible matrix effects for fluoxastrobin (HEC-5725) and HEC 5725-deschlorophenyl were eliminated by using internal standards solution of isotopically labeled reference items. For the HEC 5725-oxazepine, the parent fluoxastrobin (HEC-5725) internal standard was used as a surrogate reference.

Two product ions per analyte were selected for monitoring; one ion mass transition served as quantitation and the other ion mass transition was used for confirmation. The mass transitions for both the quantitation as well as the confirmation are presented below:

Analyte	Quantitation MRM[a]	Confirmation MRM[a]
Fluoxastrobin (HEC-5725)	459.2 → 188.1	459.2 → 427.1
Fluoxastrobin (HEC-5725) Internal Standard (IS)	463.2 → 188.1	463.2 → 431.1
HEC 5725-deschlorophenyl	349.2 → 102.1	349.2 → 317.1
HEC 5725-deschlorophenyl Internal Standard (IS)	353.1 → 102.1	353.1 → 321.1
HEC 5725-oxazepine	409.2 → 169.1	409.2 → 365.1

[a] All masses reported in amu

3.5. INSTRUMENTATION

Example LC/MS/MS conditions are presented in Appendix 3.

3.6. CALCULATIONS

The example calculations displayed below were used by the laboratory performing the independent laboratory validation. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Fluoxastrobin (HEC-5725) residues were quantified using internal standard linear regression analysis. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by 1/x weighted linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in (ppb) using Thermo LCQuan Software (Version 2.9), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients for slope, M, and intercept, B, for each analytical set. The calibration standards were fit to the linear equation:

$$Y = MX + B$$

where: X is the concentration of the analyte residue found in ng/mL[a]
M is the calibration line slope
B is the calibration line intercept
Y is the (native peak area / isotopic peak area) ratio

[a] As the reference standards and samples contain the same internal standard concentrations and sample equivalence concentrations, this value also is analyte residue of ng/g (ppb)

The equation shown below is used to calculate of residues. After regression coefficients were calculated, residues in ng/g (ppb) are determined using the following equation,

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

The percent recovery was calculated as follows:

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

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

4.5. METHOD DETECTION LIMIT (MDL)

An estimate of the potential method detection limit (MDL) was determined by examining the variability in the recovery as measured by the standard deviation of the residue amounts found at the target LOQ fortification.

The estimated potential method detection limit was calculated using the equation shown below.

MDL (calculated) = (standard deviation x $t_{0.99}$)

Where $t_{0.99}$ = one-tailed t-statistic at the 99% confidence level for n-1 replicates.

As five replicate analyses were performed during verification, from the Student t-tables, $t_{0.99}$ = 3.747.

The calculated MDL (ng/g) for fluoxastrobin (HEC-5725) Quantitation MRM was determined to be 0.0050 ppb as presented in Table 7. The calculated MDL (ng/g) for fluoxastrobin (HEC-5725) Confirmation MRM was determined to be 0.0043 ppb as presented in Table 8. The calculated MDL (ng/g) for HEC 5725-deschlorophenyl Quantitation MRM was determined to be 0.0062 ppb as presented in Table 7. The calculated MDL (ng/g) for HEC 5725-deschlorophenyl Confirmation MRM was determined to be 0.0070 ppb as presented in Table 8. The calculated MDL (ng/g) for HEC 5725-oxazepine Quantitation MRM was determined to be 0.0096 ppb as presented in Table 7. The calculated MDL (ng/g) for HEC 5725-oxazepine Confirmation MRM was determined to be 0.0067 ppb as presented in Table 8.

Appendix 3. Typical HPLC-MS/MS Parameters

HPLC Conditions:
Autosampler ID: Dionex Ultimate 3000 XRS Serial # 330573

Pump ID: Ultimate 3000 XRS LPG-3400-XRS Serial # 32073

Column Heater: Dionex Serial # 6002689
Quantitation Software Version: LCquan 2.9

Column make: Phenomenex Kinetex C18 (Ser. No.: H17-270287)
Column Measurements: 1.7 µm, 100Å, 100 x 2.1 mm
Column Temperature (°C): 40 °C

Injection Volume (µL): 60
Loop Volume (µL): 50
Aqueous Mobile Phase ID: 0.1% Formic Acid in Optima LC/MS Water
Oganic Mobile Phase ID: 0.1% Formic Acid in Optima LC/MS Methanol

Syringe Wash Solvent 2: MeOH
Syringe Wash Solvent 1: ACN/water (1:9) with 0.1% Acetic Acid
Solvent Profile:

Time (min)	% Aqueous	% Organic	Flow Rate (mL/min)
0.0	95	5	0.30
0.1	95	5	0.30
6.0	5	95	0.30
7.0	5	95	0.30
8.0	95	5	0.30
11.0	End		

Mass Spectrometer Conditions
Instrument ID: TSQ Quantiva Serial # TQH-Q1-0276

Global Settings
Method Duration (min) =11
Ion Source Type = H-ESI
Spray Voltage: Positive Ion (V) = 3500
Spray Voltage : Negative Ion (V) = 2500
Sheath Gas (Arb) = 40
AuxGas (Arb) = 12
SweepGas (Arb) = 1
Ion Transfer Tube Temp (°C) = 333
Vaporizer Temp (°C) = 317
APPI Lamp = Not in use
Experiment Type: SRM
Use Cycle Time: False
Chrom Filter (sec): 5.0

Data Mode: Centroid
Collision Gas Pressure (mTorr): 1.5
Use Calibrated RF Lens: False
Q1 Resolution (FWHM): 0.7
Q3 Resolution (FWHM): 0.7
Source Fragmentation (V): 10.0

Compound Name	Start Time (min)	End Time (min)	Polarity	Pre-cursor (m/z)	Prod-uct (m/z)	Collision Energy (V)	Dwell Time (msec)	RF Lens
Deschlorophenyl	3.5	5.25	Positive	349.2	102.1	48	100	69
Deschlorophenyl Conf	3.5	5.25	Positive	349.2	317.1	16	100	69
Deschlorophenyl-IS	3.5	5.25	Positive	353.1	102.1	48	100	69
Deschlorophenyl-IS Conf	3.5	5.25	Positive	353.1	321.1	16	100	69
Oxazepine	5.26	9	Positive	409.2	169.1	36	75	109
Oxazepine Conf	5.26	9	Positive	409.2	365.1	26	75	109
Fluoxastrobin	5.26	9	Positive	459.2	188.1	35	75	83
Fluoxastrobin Conf	5.26	9	Positive	459.2	427.1	18	75	83
Fluoxastrobin-IS	5.26	9	Positive	463.2	188.1	35	75	83
Fluoxastrobin-IS Conf	5.26	9	Positive	463.2	431.1	18	75	83

Appendix 4 Example Calculations

All residues were quantified using linear regression analysis with LCQuan Software, a computer-programmed data capturing system. The LCQuan Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set. A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. For fluoxastrobin (HEC-5725), a calibration curve was generated by linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in (ppb).

Fluoxastrobin (HEC-5725)

The fluoxastrobin (HEC-5725) standards were fit to the linear equation: Y = MX + B

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

The slope and intercept were obtained from the calibration curve generated by LCQuan, and are presented in Appendix 1. The calibration points were weighted 1/x to provide better fit near the limit of detection.

The example is for the calculation of fluoxastrobin (HEC-5725) quantitation residues for sample [Fluox-Met-ILV-0.05-ppb-LOQ-QC-06] (set JDD170920A) which was fortified to 0.05 ppb of fluoxastrobin (HEC-5725). This chromatogram is presented in Appendix 2.

After regression coefficients were calculated, the residue in ppb was determined. The ppb of fluoxastrobin (HEC-5725) in water was calculated using the following equation:

$$\text{Fluoxastrobin (HEC-5725) found (ppb)} = \frac{(Y-B)}{M}$$

Fluoxastrobin (HEC-5725) Native Peak Area (Native)	Fluoxastrobin (HEC-5725) IS Peak Area (IS)	
19,163	74,367	
[Y] = (Native/IS)	[B]	[M]
0.2576815	0.0216684	5.34479

$$\text{Fluoxastrobin (HEC-5725) found} = \frac{(0.2576815 - [0.0216684])}{5.34479} = 0.04416 \text{ ppb}$$

Therefore sample [sample] contains 0.04416 ppb fluoxastrobin (HEC-5725) which agrees with the value reported in the raw data.

The percent recovery was calculated in Excel as follows:

$$\text{Recovery \%} = \frac{R}{T} X\,100$$

Where: R = ppb of target analyte found in fortified samples
T = theoretical ppb in fortified sample

0.04416 ppb of fluoxastrobin (HEC-5725) was detected in water sample [sample] (R); and the sample was fortified to 0.05 ppb fluoxastrobin (HEC-5725) (T).

Therefore:

$$\text{Fluoxastrobin (HEC-5725) \% Recovery} = \frac{0.04416}{0.05} \times 100 = 88\%$$