

**INDEPENDENT LABORATORY VALIDATION OF
“DETERMINATION OF JAU 6476, DESTHIO (SSX 0665),
S-METHYL, AND THIAZOCINE IN WATER BY LC/MS/MS”**

SUMMARY

An independent laboratory validation (ILV) of “Determination of JAU 6476, Desthio (SSX 0665), S-methyl, and thiazocine in Water by LC/MS/MS”, method number 200517, provided by Bayer, was conducted in accordance with OPPTS 850.7100. The method validation was conducted by first fortifying 50-mL samples of water with JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine and then fortifying with stable isotope internal standards. The analytes were extracted from water with C18 solid phase extraction (SPE) cartridge and eluted with acetonitrile/water (9/1, v/v). Analysis was performed using high performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

1.0 PURPOSE

The objective of this study was to conduct a GLP independent laboratory validation of Bayer Method “Determination of JAU 6476, Desthio (SSX 0665), S-methyl, and thiazocine in Water by LC/MS/MS” to meet regulatory requirements as detailed in OPPTS 850.7100, Data Reporting for Environmental Chemistry Methods. This study was conducted in accordance with U.S. Environmental Protection Agency (40 CFR Part 160) Federal Insecticide, Fungicide and Rodenticide Act, Good Laboratory Practice Standards; Final Rule.

2.0 METHOD SUMMARY

The validation procedure followed Bayer Method “Determination of JAU 6476, Desthio (SSX 0665), S-methyl, and thiazocine in Water by LC/MS/MS.” Residues of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine were extracted from 50 mL of control water. A sample (50 mL) of water was measured with a 50-mL graduated cylinder. The water was then fortified as appropriate with the test substances. An aliquot (100 µL) of the 3.0 µg/mL internal standard solution was added to the cylinder. The cylinder was shaken briefly to mix. The water was passed through a preconditioned 500-mg C₁₈ SPE cartridge. The cylinder was rinsed with 5 mL of HPLC-grade water and the rinse was added to the cartridge. The

water was allowed to drain and the cartridge was allowed to dry for 1-2 minutes after being purged with nitrogen. The cartridge was eluted with 4 mL of acetonitrile: water (9:1, v/v) into a 13-mL centrifuge tube. A portion (~ 2 mL) of the sample was transferred to a HPLC autosampler vial. Analysis was performed using LC-MS/MS. A flow chart for the method can be found in Figure 1.

The residues of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine were calculated from the relative response factor between the native analyte and a deuterated analog (internal standard) and the slope and intercept obtained from a 1/x-weighted linear least squares regression of the relative responses obtained from five calibration standards. Micromass MassLynx software was used to determine chromatographic peak areas and calculate concentrations. Microsoft Excel® 2000 was used for statistical calculations including averages and standard deviations.

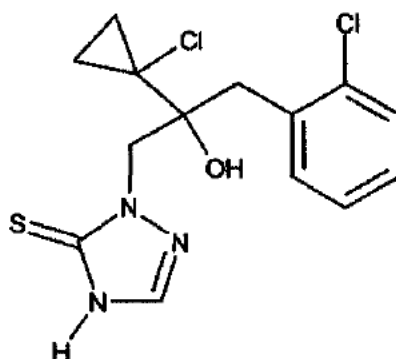
3.0 MATERIALS AND METHODS

3.1 Test and Reference Substances

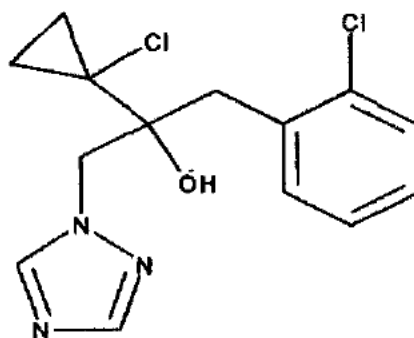
The test substances for this study were JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine. The reference substances for this study were the deuterated-analogs of the test substances, JAU 6476-triazole-1,2,4-¹⁵N,3,5-¹³C, Desthio-triazole-1,2,4-¹⁵N,3,5-¹³C₂, S-methyl JAU 6476-methyl-d₃-¹³C, and JAU 6476-thiazocine-¹⁵N₃-¹³C₂. The test and reference substances were received at room temperature from Bayer Agricultural Division, Stilwell, KS on December 5, 2002. The test/reference substances were stored at ≤-20 °C. Documentation of the synthesis as well as chemical and physical characterizations of the test/reference substances is maintained by Bayer. The chemical names, molecular weights, lot numbers (ID), purity, and chemical structures of the test substances are presented below:

Common Name:	JAU 6476
Chemical Name (CAS):	2-[2-(1-Chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-3H-1,2,4-triazole-3-thione
CAS Number:	178928-70-6
Empirical Formula:	C ₁₄ H ₁₅ Cl ₂ N ₃ OS
Molecular Weight:	344.27 g/mol
ID No.:	K-879
Purity:	99.7%

Structure:



Common Name:	SXX 0665 (Desthio)
Chemical Name:	1H-1,2,4-triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-[(2-chlorophenyl)methyl]
CAS Number:	120983-64-4
Empirical Formula:	C ₁₄ H ₁₅ Cl ₂ N ₃ O
Molecular Weight:	312.20 g/mol
ID No.:	K-1096
Purity:	98.5%
Structure:	



Common Name:	KTS 9473 (S-methyl)
Chemical Name (CAS):	1H-1,2,4-triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-[(2-chlorophenyl)methyl]-3-(methylthio)
CAS Number:	None Given

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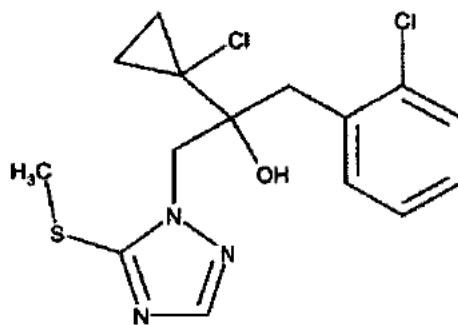
Empirical Formula: $C_{15}H_{17}Cl_2N_3OS$

Molecular Weight: 358.28 g/mol

ID No.: K-880

Purity: 98.9%

Structure:



Common Name: JAU 6476-thiazocine

Chemical Name: 5H-[1,2,4]triazolo[5,1-b][1,3]benzothiazocin-6-ol, 6-(1-chlorocyclopropyl)-6,7-dihydro-

CAS Number: None Given

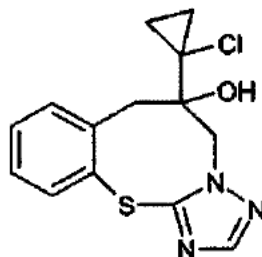
Empirical Formula: $C_{14}H_{14}N_3ClOS$

Molecular Weight: 307.80 g/mol

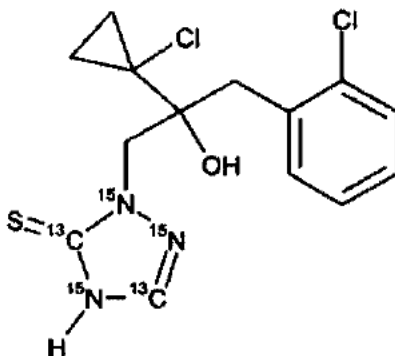
ID No.: K-972

Purity: 97.3%

Structure:

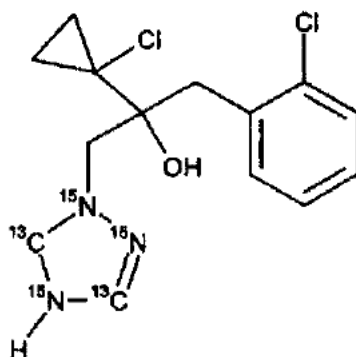


Common Name:	JAU 6476-triazole-1,2,4- ¹⁵ N,3,5- ¹³ C
Chemical Name (CAS):	3H-1,2,4-triazole-3-thione, 1-[2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-triazole-1,2,4- ¹⁵ N-3,5- ¹³ C
CAS Number:	None Given
Empirical Formula:	C ₁₂ ¹³ C ₂ H ₁₅ Cl ₂ ¹⁵ N ₃ OS
Molecular Weight:	349.24 g/mol
ID No.:	K-1098
Purity:	99.3%
Structure:	

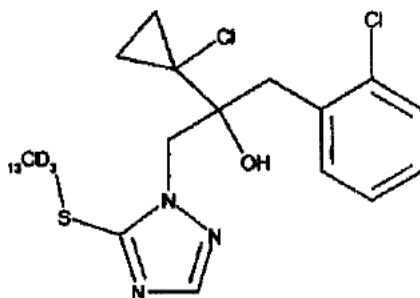


Common Name:	Desthio-triazole-1,2,4- ¹⁵ N ₃ ,3,5- ¹³ C ₂
Chemical Name:	1H-1,2,4-triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-[(2-chlorophenyl)methyl]-triazole-1,2,4- ¹⁵ N,3,5- ¹³ C
CAS Number:	None Given
Empirical Formula:	C ₁₂ ¹³ C ₂ H ₁₅ Cl ₂ ¹⁵ N ₃ O
Molecular Weight:	317.18 g/mol
ID No.:	K-893
Purity:	98.7%

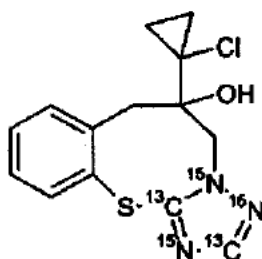
Structure:



Common Name:	S-methyl JAU 6476-methyl-d ₃ - ¹³ C
Chemical Name (CAS):	1H-1,2,4-triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-[(2-chlorophenyl)methyl]-3-(methylthio)-1,2,4- ¹⁵ N,3,5- ¹³ C
CAS Number:	None Given
Empirical Formula:	C ₁₃ ¹³ C ₁ H ₁₄ D ₃ Cl ₂ N ₃ OS
Molecular Weight:	362.29 g/mol
ID No.:	K-895
Purity:	99.8%
Structure:	



Common Name:	JAU 6476-thiazocine- ¹⁵ N ₁ - ¹³ C ₂
Chemical Name:	5H-[1,2,4]triazolo[5,1-b][1,3]benzothiazocin-6-ol, 6-(1-chlorocyclopropyl)-6,7-dihydro- ¹⁵ N ₃ , ¹³ C ₂
CAS Number:	None Given
Empirical Formula:	C ₁₄ ¹³ C ₂ H ₁₄ ¹⁵ N ₃ ClOS
Molecular Weight:	312.76 g/mol
ID No.:	K-988
Purity:	95.2%
Structure:	



3.2 Procedure of Administration of the Test Substance

The test substance was applied to the test system as an analytical standard solution by adjustable volume pipet to ensure precise delivery of a small amount of test substance.

3.3 Test System

The test system was surface water from an Arkansas rice aquatic dissipation study. The water was received at room temperature from Bayer, Stilwell, Kansas on March 17, 2003. The water test system was stored refrigerated at 1-9 °C for the duration of this study. The water was assigned a unique Battelle identifier, 03007-3-1, for use on this study.

3.4 Fortification

Samples of water (50 mL) in 50-mL graduated cylinders were fortified in replicates of five with the target amount of JAU 6476, Dethio, S-methyl, and JAU 6476-thiazocine as shown below.

Level	Stock Concentration (µg/mL)	Fortification Volume (µL)	Final Concentration in Water (ppb)
Control	NA	NA	NA
1X	0.3	50	0.3
10X	0.3	500	3.0

3.5 Analytical Method

The linearity assessment and recovery data for the study was generated using the method described in the Bayer method, "Determination of JAU 6476, Desthio (SSX 0665), S-methyl, and thiazocine in Water by LC/MS/MS." A method trial sample set for water consisted of one reagent blank, two control (unfortified) water samples, five replicate fortifications at the LOQ (0.3 ppb), and five replicate fortifications at ten times the LOQ (3.0 ppb). A total of thirteen samples were processed for a validation trial.

A sample (50 mL) of water was measured with a 50-mL graduated cylinder. The water was then fortified as appropriate with the test substances. An aliquot (100 µL) of the 3.0 µg/mL mixed internal standard solution was added to the cylinder. The cylinder was shaken briefly to mix. The water was passed through a preconditioned 500-mg C₁₈ SPE cartridge at a rate of 10-20 mL/minute. The cylinder was rinsed with 5 mL of HPLC-grade water and the rinse was added to the cartridge. The water was allowed to drain and the cartridge was allowed to dry for 1-2 minutes after being purged with nitrogen. The cartridge was eluted with 4 mL of acetonitrile: water (9:1, v/v) into a 13-mL centrifuge tube. A portion (~ 2 mL) of the sample was transferred to a HPLC autosampler vial. Analysis was performed by HPLC-MS/MS (Figure 1).

Prior to the method validation trial, instrumental analysis parameters were optimized, linearity was assessed, and the control matrix was evaluated for the presence of interferences. To assess linearity, five calibration standards of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine were prepared in solvent using isotope-labeled internal standard and 100 µL of each calibration standard was injected in triplicate. Each standard solution contained 6.0 ppb of each internal reference standard (JAU 6476-triazole-1,2,4-¹⁵N,3,5-¹³C, Desthio-triazole-1,2,4-¹⁵N,3,5-¹³C₂, S-methyl JAU 6476-methyl-d₃-¹³C, and JAU 6476-thiazocine-¹⁵N₃-¹³C₃). The concentrations of native analyte were those listed in Method Section 3.0 and ranged from 0.15 ppb to 18 ppb. Concentrations were expressed in terms of sample equivalent.

Duplicate blank matrix samples were also prepared and analyzed with the calibration standards. The samples were processed using the same procedure that was used for the subsequent fortification trial. The blank control sample extracts prepared for the QC check were not fortified with the test substance.

3.6 Protocol Amendments and Deviations

There were no protocol amendments and no protocol deviations for this study.

3.7 Modifications/Observations for the Method Described in Bayer Method 200517

The following is a list of observations and modifications that were noted while validating the method.

Section 3.0. The HPLC autosampler differs from the original method. A HPI 100 was used instead of a ThermoFinnigan P-4000 pump and degasser and A-3000 autosampler.

Section 3.0. The LC-MS/MS equipment differs from the original method. A Micromass Quattro LC was used instead of a ThermoFinnigan TSQ 7000.

3.8 LC-MS/MS System

The following conditions, based on Section 3.0 of the supplied method, were used for LC-MS/MS analysis of the JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine.

Equipment and Conditions

HPLC:	Hewlett Packard Model 1100	
Column:	Luna C ₁₈ (2), 100 mm x 4.6 mm, 5 μm	
Column Temperature:	40 °C	
Mobile Phase A:	0.1% formic acid in water	
Mobile Phase B:	0.1% formic acid in acetonitrile	
Flow Rate:	800 μL/minute	
Gradient:	0.0 minutes	45% B
	5.0 minutes	95% B
	7.5 minutes	95% B
	7.6 minutes	45% B
	9.0 minutes	45% B
	12.0 minutes	45% B
Injection Volume:	100 μL	
Run Time:	Approximately 12 minutes	
Retention Time:	JAU 6476	~ 5.8 minutes
	Desthio	~ 5.2 minutes
	S-methyl	~ 7.0 minutes
	JAU 6476-thiazocine	~ 3.8 minutes
Mass Spectrometer:	Micromass Quattro LC	

Ionization:	Electrospray, Positive Ion Mode (ESP+)	
Ions Monitored:	JAU 6476	m/z 344>326
	JAU 6476 (IS)	m/z 349>331
	Desthio	m/z 312>70
	Desthio (IS)	m/z 317>75
	S-methyl	m/z 358>116
	S-methyl (IS)	m/z 362>120
	JAU 6476-thiazocine	m/z 308>190
	JAU 6476-thiazocine (IS)	m/z 313>195
Source Temperature:	90 °C	
Drying Gas:	Nitrogen	
Cone Voltage:	JAU 6476	15 V
	Desthio	30 V
	S-methyl	30 V
	JAU 6476-thiazocine	40 V
Collision Gas:	Argon	
Collision Energy:	JAU 6476	10 eV
	Desthio	25 eV
	S-methyl	20 eV
	JAU 6476-thiazocine	25 eV
Multiplier:	750 V	

3.9 Data Calculations/Statistical Methods

3.9.1 Linearity Assessment

The linearity assessment of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine was determined by using calibration curves obtained from the peak area ratio between the native analytes and their deuterated-analogs (internal standards) at five sample-equivalent analyte concentrations, with three injections per concentration. The sample-equivalent analyte concentrations were 0.15 ppb, 0.3 ppb, 1.8 ppb, 6.0 ppb, and 18.0 ppb. The internal standard concentration was 6.0 ppb in all standard solutions. The results of the linearity assessment can be found in Tables 1-4. The standard curves can be found in Appendix I.

The ion chromatogram peaks for each analyte and its internal standard were integrated for each calibration level. The relative response ratio of each standard was calculated using the following equation.

$$\text{Relative Response Ratio} = \frac{(\text{Peak Area STD})}{(\text{Peak Area IS})}$$

The calibration curves were constructed by plotting the relative response ratio of each standard versus the amount, in ppb, of the native analyte concentration. Linearity was considered acceptable if the coefficient of determination was equal to or greater than 0.99. Results obtained from the analysis of the calibration solutions are shown in Tables 1-4.

3.9.2 Method Validation Trial

The concentrations of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine present in the fortified samples were determined by using a standard curve obtained from the peak area ratio between the native analyte and its deuterated internal standard, at five sample-equivalent concentrations, with three injections per concentration. The standard injections were interspersed between the fortified sample extracts in the sample analysis set.

The calibration curve for each test substance was constructed by plotting the 1/x-weighted peak area ratio of each standard versus the concentration of the standard injected. The y-intercept and slope of the standard curve were used to calculate the concentration of each test substance in the samples as follows:

$$\text{Peak Area Ratio} = \frac{\text{Area of Native Analyte}}{\text{Area of Deuterated Internal Standard}}$$
$$\text{Residue Level (ppb)} = \frac{(\text{Sample Peak Area Ratio} - \text{Intercept})}{\text{Slope}}$$

The method recovery was calculated by dividing the determined residue level by the fortification concentration and multiplying by 100. The following formula was used:

$$\text{Method Recovery (\%)} = \frac{\text{Residue Level (found)}}{\text{Fortification Level}} \times 100$$

For example, the concentration of JAU 6476 in water sample 03007-12-5, fortified at 0.3 ppb, was calculated as follows:

$$\text{Peak Area Ratio} = \frac{2770}{52476} = 0.053$$

$$\text{JAU 6476 Residue Level (ppb)} = \frac{(0.053 - 0.00273877)}{0.153797} = 0.325 \text{ ppb}$$

and

$$\text{Method Recovery (\%)} = \frac{0.325 \text{ ppb}}{0.30 \text{ ppb}} \times 100 = 108.3\%$$

Figure 1. Flowchart for the JAU 6476, Desthio, S-Methyl, and JAU 6476-Thiazocine in Water Extraction Method

