

**INDEPENDENT LABORATORY VALIDATION OF “ANALYSIS OF  
JAU 6476, DESTHIO, S-METHYL, AND JAU 6476-THIAZOCINE IN SOIL”**

**SUMMARY**

An independent laboratory validation (ILV) of “Analysis of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine in Soil”, method number 200515, provided by Bayer, was conducted in accordance with OPPTS 850.7100. The method validation was conducted by fortifying samples of soil with JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine. The analytes were extracted from (15 g) soil with 30 mL of acetonitrile/water/cysteine hydrochloride (800/200/0.5, v/v/w) at room temperature in a mechanical shaker for one hour. The sample was then centrifuged at approximately 2300 rpm for approximately 10 minutes. The extract (4 mL) was transferred to a culture tube and stable isotope internal standards were added. An aliquot of the extract (700 µL) was placed in an HPLC autosampler vial and diluted with 300 µL of water. 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 (ILV) of Bayer Method “Analysis of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine in Soil” 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, and Good Laboratory Practice Standards; Final Rule.

**2.0 METHOD SUMMARY**

The validation procedure followed Bayer Method “Analysis of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine in Soil.” Residues of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine were extracted from 15 g of homogenized control soil with acetonitrile/water/cysteine hydrochloride (800/200/0.5, v/v/w) by shaking at room temperature for approximately 1 hour. The sample was then centrifuged at approximately 2300 rpm for approximately 10 minutes. A portion (4 mL) of the extract

was transferred to a separate culture tube and the sample was fortified with deuterated internal standard. An aliquot (700  $\mu$ L) of the extract was transferred to an HPLC autosampler vial. Water (300  $\mu$ L) was added to the extract and the sample was mixed. 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.

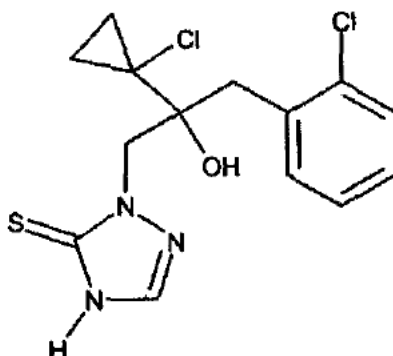
### 3.0 MATERIALS AND METHODS

#### 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-<sup>15</sup>N,3,5-<sup>13</sup>C, Desthio-triazole-1,2,4-<sup>15</sup>N,3,5-<sup>13</sup>C<sub>2</sub>, S-methyl JAU 6476-methyl-d<sub>3</sub>-<sup>13</sup>C, and JAU 6476-thiazocine-<sup>15</sup>N<sub>3</sub>-<sup>13</sup>C<sub>2</sub>. The test and reference substances were received at room temperature from Bayer Agricultural Division, Stilwell, KS on December 05, 2002. The test/reference substances were stored at approximately  $\leq$ -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:

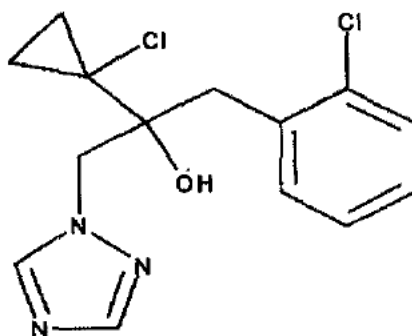
Common Name:	JAU 6476
Chemical Name (CAS):	2-[2-(1-Chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-3H-1,2,4-triazole-3-thione
CAS Number:	178928-70-6
Empirical Formula:	C <sub>14</sub> H <sub>15</sub> Cl <sub>2</sub> N <sub>3</sub> OS
Molecular Weight:	344.27 g/mol
ID No.:	K-879
Purity: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

Structure:



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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 <sub>14</sub> H <sub>15</sub> Cl <sub>2</sub> N <sub>3</sub> O
Molecular Weight:	312.20 g/mol
ID No.:	K-1096
Purity:	98.5%
Structure:	



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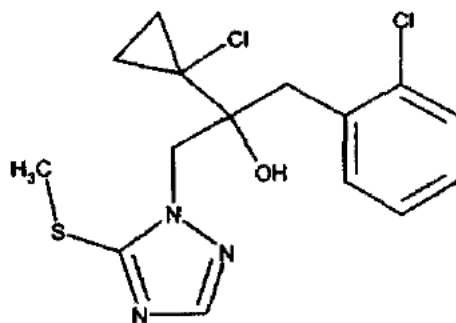
Empirical Formula:  $C_{15}H_{17}Cl_2N_3OS$

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

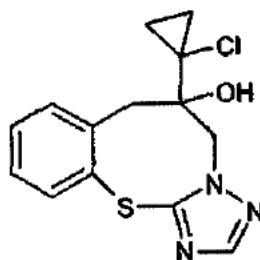
Empirical Formula:  $C_{14}H_{14}N_3ClOS$

Molecular Weight: 307.80 g/mol

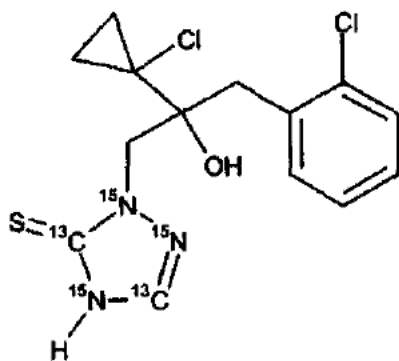
ID No.: K-972

Purity: 97.3%

Structure:



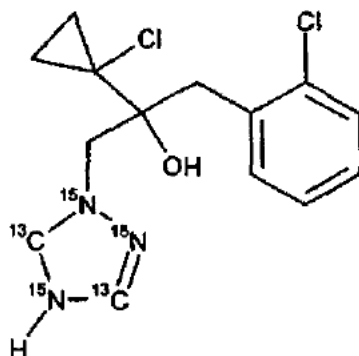
Common Name:	JAU 6476-triazole-1,2,4- <sup>15</sup> N,3,5- <sup>13</sup> C
Chemical Name (CAS):	3H-1,2,4-triazole-3-thione, 1-[2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-triazole-1,2,4- <sup>15</sup> N-3,5- <sup>13</sup> C
CAS Number:	None Given
Empirical Formula:	C <sub>12</sub> <sup>13</sup> C <sub>2</sub> H <sub>15</sub> Cl <sub>2</sub> <sup>15</sup> N <sub>3</sub> OS
Molecular Weight:	349 g/mol
ID No.:	K-1098
Purity:	99.3%
Structure:	



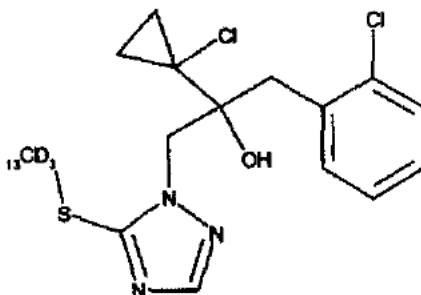

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Common Name:	Desthio-triazole-1,2,4- <sup>15</sup> N <sub>3</sub> ,3,5- <sup>13</sup> C <sub>2</sub>
Chemical Name:	[1H-1,2,4-triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-[(2-chlorophenyl)methyl]-triazole-1,2,4- <sup>15</sup> N,3,5- <sup>13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

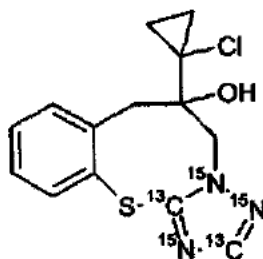
Structure:



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



Common Name:	JAU 6476-thiazocine- <sup>15</sup> N <sub>3</sub> - <sup>13</sup> C <sub>2</sub>
Chemical Name:	5H-[1,2,4]triazolo[5,1-b][1,3]benzothiazocin-6-ol, 6-(1-chlorocyclopropyl)-6,7-dihydro- <sup>15</sup> N <sub>3</sub> , <sup>13</sup> C <sub>2</sub>
CAS Number:	None Given
Empirical Formula:	C <sub>14</sub> <sup>13</sup> C <sub>2</sub> H <sub>14</sub> <sup>15</sup> N <sub>3</sub> 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 control soil from Manitoba. This soil was chosen for this study because it was considered to be a more difficult soil because of a higher than typical percent organic matter. It was also chosen because triazole recoveries from this Manitoba soil were consistently lower than from any other soil type. The soil was received at room temperature from Bayer, Stilwell, Kansas on December 05, 2002. The soil test system was stored frozen at  $\leq -15$  °C for the duration of this study. The soil was assigned a unique Battelle identifier, 03002-03-01 for use on this study.

### 3.4 Fortification

Samples of soil (15 g) in 50-mL centrifuge tubes were fortified in replicates of five with 150  $\mu$ L of acetonitrile/water/cysteine hydrochloride (980/20/0.01, v/v/w) and the target amount of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine as shown below.

Level	Stock Concentration ( $\mu$ g/mL)	Fortification Volume ( $\mu$ L)	Final Concentration in Soil (ppb)
Control	NA	NA	NA
1X	1	150	10
10X	10	150	100

### 3.5 Analytical Method

The linearity assessment and recovery data for the study was generated using the method described in the Bayer method, "Analysis of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine in Soil." A method trial sample set for soil consisted of one reagent blank, two control (unfortified) soil samples, five replicate fortifications at the LOQ (10 ppb), and five replicate fortifications at ten times the LOQ (100 ppb). A total of thirteen samples were processed for a validation trial.

A sample (15 g) of soil was weighed into a 50-mL centrifuge tube. The soil was then fortified as appropriate with the test substances. The sample was extracted with 30 mL of acetonitrile/water/cysteine hydrochloride (800/200/0.5, v/v/w) by shaking in a horizontal position with a mechanical shaker for approximately one hour at room temperature. The sample was then centrifuged for approximately 10-15 minutes at approximately 2300 rpm. A portion (4 mL) of the sample was transferred to a separate tube. An aliquot (40  $\mu$ L) of the 10  $\mu$ g/mL internal standard solution was added to the tube and mixed briefly. An aliquot of the extract (700  $\mu$ L) containing internal standard was transferred to an HPLC autosampler vial. Water (300  $\mu$ L) was added to the autosampler vial and the vial was briefly mixed. 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  $\mu$ L of each injected in triplicate. Each standard solution contained 200 ppb of each internal reference standard (JAU 6476-triazole-1,2,4-<sup>15</sup>N,3,5-<sup>13</sup>C, Desthio-triazole-1,2,4-<sup>15</sup>N<sub>3</sub>,3,5-<sup>13</sup>C<sub>2</sub>, S-methyl JAU 6476-methyl-d<sub>3</sub>-<sup>13</sup>C, and JAU 6476-thiazocine-<sup>15</sup>N<sub>3</sub>-<sup>13</sup>C<sub>2</sub>). The concentrations of native analyte were those listed in Method Section 3.0 and ranged from 5 ppb to 200 ppb. Concentrations were expressed in terms of sample equivalent.



Duplicate blank matrix samples were also prepared and analyzed with a curve. The samples were processed using the same procedure that was used for the 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 was one protocol amendment for this study. The manager was changed from A. M. Bloomberg to R. Allen due to the original manager changing positions within Bayer CropScience.

### 3.7 Modifications/Observations for the Method Described in Bayer Method 200515

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

Section 4.0. The HPLC autosampler differs from the original method. A HP1100 was used instead of a ThermoFinnigan P-4000 pump and degasser and A-3000 autosampler.

Section 4.0. The LC-MS/MS equipment differs from the original method. A Micromass Quattro LC was used instead of a ThermoFinnigan TSQ 7000. Two separate injections were required in order to achieve the required sensitivity for JAU 6476-thiazocine. The JAU 6476-thiazocine required a much lower collision gas than the other three compounds. This parameter cannot be adjusted in a single injection and must be manually changed between acquisition methods.

### 3.8 LC-MS/MS System

The following conditions, based on Section 4.0 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 <sub>18</sub> (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:	400 μL/minute

Gradient:	0.0 minutes	45% B
	3.0 minutes	95% B
	5.0 minutes	95% B
	7.5 minutes	95% B
	7.6 minutes	45% B
	10.0 minutes	45% B
Injection Volume:	100 $\mu$ L	
Run Time:	Approximately 12 minutes	
Retention Time:	JAU 6476	~ 7.5 minutes
	Desthio	~ 7.3 minutes
	S-methyl	~ 9.0 minutes
	JAU 6476-thiazocine	~ 6.0 minutes
Mass Spectrometer:	VG/Fisons, 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 $^{\circ}$ C	
Drying Gas:	Nitrogen	
Cone Voltage:	JAU 6476	18 V
	Desthio	38 V
	S-methyl	40 V
	JAU 6476-thiazocine	45 V
Collision Gas:	Argon at $\sim 1.5\text{e-}3$ for JAU 6476, Desthio, and S-methyl Argon at $\sim 1.8\text{e-}4$ for JAU 6476-thiazocine	
Collision Energy:	JAU 6476	10 eV
	Desthio	45 eV
	S-methyl	40 eV
	JAU 6476-thiazocine	50 eV

Multipliers: 800 V for JAU 6476, Desthio, and S-methyl  
900 V for JAU 6476-thiazocine

### 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 standard curves obtained from the peak area ratio between the native analyte and their deuterated-analogs (internal standards) at five standard concentrations, with three injections per concentration. The standard concentrations were 5 ppb, 10 ppb, 50 ppb, 100 ppb, and 200 ppb. The internal standard concentration was 200 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 standard ion peak and the internal standard ion peak were integrated for each standard 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 standard injected. Linearity was considered acceptable if the coefficient of determination was equal to or greater than 0.99. Results of the calibration curves are shown in Tables 1-4.

#### 3.9.2 Method Validation Trial

The residues of JAU 6476, Desthio, S-methyl, and JAU 6476-thiazocine in the fortified samples were quantified based on the relative response ratios of the analyte peak plotted against the standard curve as shown below. The slope and intercept from a weighted (1/X) linear regression curve was used for quantitation.

$$\text{Slope X Standard Concentration (ppb) + y intercept} = \frac{\text{Native Area}}{\text{Internal Standard Area}}$$

The method recovery was calculated by dividing the determined concentration in ppb (ng/kg) by the expected concentration of the analyte in the sample and multiplying that ratio by 100. The following formula was used:

$$\text{Recovery (\%)} = \frac{\text{Fortified Sample Conc. (ppb)}}{\text{Fortification Level}} * 100$$