Analysis of JAU 6476, Desthio, S-methyl and JAU 6476-thiazocine in water

1.0 SUMMARY

An analytical method was developed to quantify JAU 6476, Desthio (SSX 0665), S-methyl and thiazocine water using high-performance liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS). The method was validated from control water obtained from New Port (Arkansas) at 0.3 ppb. Isotopic internal standards of JAU 6476, Desthio (SSX 0665), S-methyl and thiazocine were added to the water samples (50 mL). The water samples were extracted with C_{18} solid phase extraction (SPE) cartridge and eluted with 4 milliliter of acetonitrile : water (9:1, v/v). The resultant solution was analyzed by LC-MS/MS, and quantitation was done against known amount of isotopic internal standards.

2.0 INTRODUCTION

JAU 6476 is an experimental fungicide being developed by the Bayer CropScience for use on wheat, corn, peanuts, barley, canola and vegetables. It belongs to the class of Triazlinthione. It is effective against leaf spots, ear stem, fusarium, bunt, smut, powdery mildew and sheath blight.

3.0 EXPERIMENTAL

3.1 Equipment (Functionality equivalents may be substituted)

- Various general laboratory glassware and utensils
- Borosilicate glass disposable culture tube, 20 x 150 mm (Fisher Scientific 14-961-33)
- HPLC vials and caps (2-mL, Wheaton #223682)
- 500 mg of C18 SPE cartridge (Varia, Part 1411-3027, 10 mL or Alltech Extract Clean Part No. 305250, 18 mL)
- Analytical Balance (Mettler A163)
- Balance, Top loader, capable of weighing to the nearest 0.01 g
- SP Vortex mixer (Baxter S8223-1)
- Luna C18(2), 100 x 4.6 mm, 5mm (Phenomenex, Part No. 00D-4252-E0)
- TSQ 7000 LC/Tandem Mass Spectrometer with ESI or APCI interface, built-in column heater and gradient HPLC, or equivalent (Finnigan Corp)

3.2 Reagents and Solvents

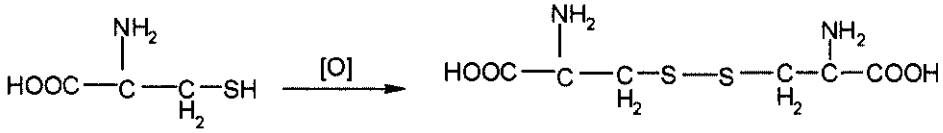
Use as a guide, equivalent reagents or solvents may be substituted.

- Methanol (MeOH; HPLC Grade, Burdick & Jackson #230-4)
- Acetonitrile (ACN; HPLC Grade, Burdick & Jackson #015-4)
- Cysteine hydrochloride ($C_3H_7NO_2S.HCl.H_3O$, Fischer Scientific, #BP376-100, CAS 7048-04-6)
- Formic acid (88%, J.T. Baker)
- Water (Self-regenerated ion-exchange Millipore water)

Solvent A : Acetonitrile / Water / Cysteine hydrochloride (980/20/0.01; v/v/w)
Add 0.01 g cysteine hydrochloride into 20 mL water. Shake until it is dissolved. Add 980 mL acetonitrile into the solvent.

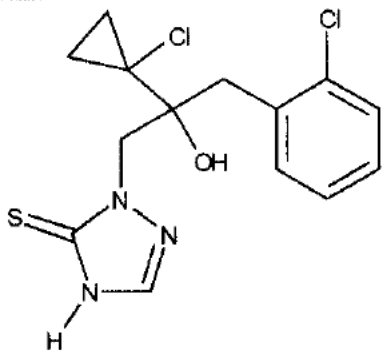
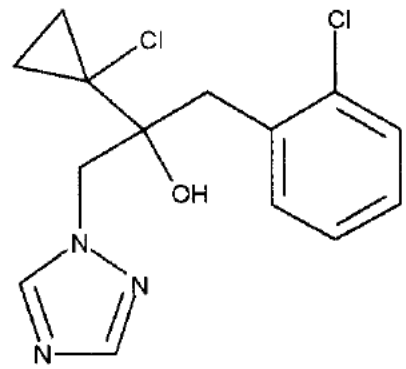
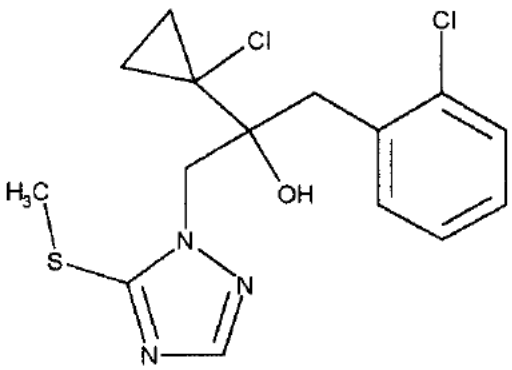
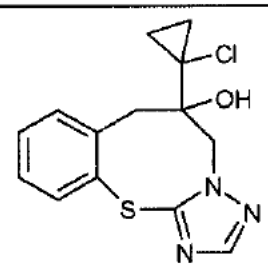
Solvent B: Acetonitrile / Water / Cysteine hydrochloride (400/600/0.1; v/v/w)
Add 0.1 g cysteine hydrochloride into 600 mL water. Shake until it is dissolved. Add 400 mL of acetonitrile into the solvent.

JAU 6476 is quite unstable and might react with oxygen in air to form other products. Since L-Cysteine hydrochloride has higher affinity to oxygen, it reacts with excess oxygen to form L-Cystine and thus minimizes the oxidation of JAU 6476.

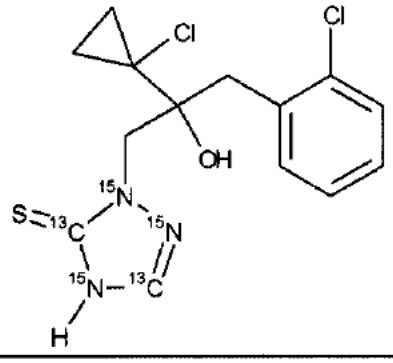
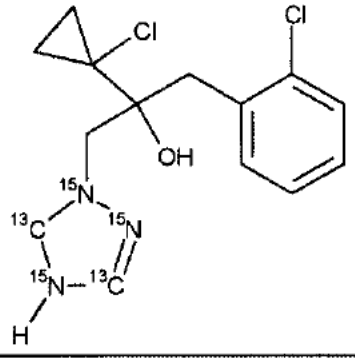
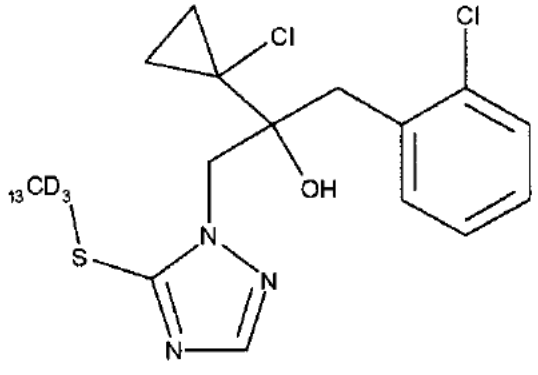
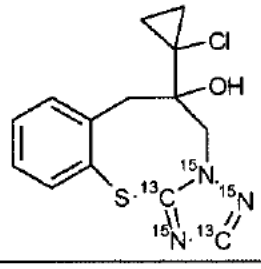


Cysteine Cystine

3.3 Structures

Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	JAU 6476 K-879 or equivalent $C_{14}H_{15}Cl_2N_3OS$ 343.0 99.4% 05/31/2002	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	SSX 0665 (Desthio) K-913 or equivalent $C_{14}H_{15}Cl_2N_3O$ 311.1 99.6% 01/16/2006	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	S-methyl K-880 or equivalent $C_{14}H_{17}Cl_2N_3OS$ 357.1 98.9% 10/27/2005	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	JAU 6476 thiazocine K-973 or equivalent $C_{14}H_{14}ClN_3OS$ 307.1 97.4% 02/20/2003	

Internal Standards

Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	JAU 6476-triazole-1,2,4-^{15}N, 3,5-^{13}C K-1098 or equivalent $C_{12}{}^{13}C_2H_{15}Cl_2{}^{15}N_3OS$ 348.03 99.4% 10/23/2006	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	Desthio-triazole-1,2,4-^{15}N, 3,5-^{13}C K-893 or equivalent $C_{12}{}^{13}C_2H_{15}Cl_2{}^{15}N_3O$ 316.1 98.7% 01/06/2005	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	S-methyl JAU 6476-triazole-1,2,4-N, 3,5-^{13}C K-895 or equivalent $C_{14}{}^{13}C_1H_{14}D_3Cl_2N_3OS$ 361.1 99.8% 01/03/2005	
Common Name Standard Ref. Empirical Formula Molecular Weight Purity Expiration Date	JAU 6476 thiazocine-^{15}N, $^{13}C_2$ K-988 or equivalent $C_{12}{}^{13}C_2H_{14}{}^{15}N_3SClO$ 312.8 95.2% 02/28/2004	

3.4 Safety and Health

The toxicity of each chemical used in this method has not been precisely determined, and thus each compound must be treated as a potential health hazard. With this in mind, exposure to these chemicals should be reduced to the lowest reasonable level by whatever means available.

3.5 Procedures

3.5.1 General Definitions

There are two definitions of standard concentration used in this method. The first is defined in terms of "µg/mL" or "ng/mL", which describes the concentration of stock solutions and fortification solutions. The second definition is in terms of "ng/mL" (ppb) of original water sample, which applies to all quantification and linearity standard solutions. This definition takes into account any aliquoting, concentration, or dilution of samples during sample preparation. Any concentration specified as or "ppb" is a sample-equivalent concentration.

"Native" is a term applied to standards or solutions containing the actual analytes in the method in order to differentiate them from isotopically labeled "Internal Standard".

3.5.1.1 Native Analyte Solutions

A stock solution of each standard, K-879 (native JAU 6476), K-913 (Desthio or SXX 0665), K-880 (S-methyl) and K-972 (JAU 6476-thiazocine) or equivalent is prepared at nominal concentration of 100 µg/mL (each) as follows:

100 µg/mL stock solution of JAU 6476 in Solvent A
e.g. Add 10 mg of JAU 6476 to 100-mL volumetric flask and dilute to the mark with Solvent A.

100 µg/mL stock solution of Desthio in acetonitrile
e.g. Add 10 mg of Desthio to 100-mL volumetric flask and dilute to the mark with acetonitrile.

100 µg/mL stock solution of S-methyl in acetonitrile
e.g. Add 10 mg of S-methyl to100-mL volumetric flask and dilute to the mark with acetonitrile.

100 µg/mL stock solution of JAU 6476-thiazocine in acetonitrile
e.g. Add 10 mg of JAU 6476-thiazocine to100-mL volumetric flask and dilute to the mark with acetonitrile.

If the standard purity is < 98%, correct the standard concentration to its absolute concentration using the standard purity. Label each flask with the standard name,

actual weight and date. Sonicate each solution for about 5 minutes. Store all solution in a freezer (< -7 °C) and protect from light when not in use.

3.5.1.2 Internal Standard Stock Solutions

A stock solution of each standard, K-1098 (JAU 6476-triazole-1,2,4-^{15}N- 3,5-^{13}C), K-893 (Desthio-triazole-1,2,4-^{15}N-3,5-^{13}C), K-895 (S-methyl JAU 6476-methyl-d_3-^{13}C) and K-988 (JAU 6476-thiazocine-1,2,4-^{15}N-3,5-^{13}C) or equivalent, is prepared at nominal concentration of 100 µg/mL (each) as follows:

100 µg/mL stock solution of JAU 6476-triazole-1,2,4-^{15}N- 3,5-^{13}C in Solvent A
e.g. Add 10 mg of JAU 6476-triazole-1,2,4-^{15}N- 3,5-^{13}C to 100-mL volumetric flask and dilute to the mark with Solvent A.

100 µg/mL stock solution of Desthio-triazole-1,2,4-^{15}N-3,5-^{13}C in acetonitrile
e.g. Add 10 mg of Desthio-triazole-1,2,4-^{15}N-3,5-^{13}C to 100-mL volumetric flask and dilute to the mark with acetonitrile.

100 µg/mL stock solution of S-methyl JAU 6476-methyl-d_3-^{13}C in acetonitrile
e.g. Add 10 mg of S-methyl JAU 6476-methyl-d_3-^{13}C to 100-mL volumetric flask and dilute to the mark with acetonitrile.

100 µg/mL stock solution of JAU 6476-thiazocine-1,2,4-^{15}N-3,5-^{13}C in acetonitrile
e.g. Add 10 mg of JAU 6476-thiazocine-1,2,4-^{15}N-3,5-^{13}C to 100-mL volumetric flask and dilute to the mark with acetonitrile.

3.5.1.3 Mixed Native or Internal Standard Solutions

Using the above native primary standards and isotopic internal standards, prepare the following mixed standards (Note: Since the stock solutions above are not exact measurements, the volumes used to prepare JauWaterMix 1-3 must be adjusted for the absolute concentration of the stock solutions to result in a near exact concentration for these solutions)

JauWaterMix 1: 3 µg/mL of mixed native standards
e.g. Add 1.5 mL of each of JAU 6476 (100 µg/mL), SXX 0665 (100 µg/mL), S-methyl (100 µg/mL) and JAU 6476 thiazocine (100 µg/mL) into a 50-mL volumetric flask. Dilute to the 50-mL mark with solvent A.

JauWaterMix 2: 0.3 µg/mL of mixed native standards
e.g. Pipet 5 mL of JauWaterMix 1 into a 50 mL volumetric flask and dilute to the mark with solvent A This will be the **native spiking solution**.

JauWaterMix 3: 3.0 µg/mL of mixed internal standards

e.g. Add 1.5 mL of each internal standard of Desthio-triazole-1,2,4-^{15}N-3,5-^{13}C (100 µg/mL), S-methyl JAU 6476-methyl-d_3-^{13}C (100 µg/mL) and JAU 6476-thiazocine-1,2,4-^{15}N-3,5-^{13}C (100 µg/mL) and JAU 6476-triazole-1,2,4-^{15}N-3,5-^{13}C (100 µg/mL) into a 50-mL volumetric flask. Dilute to the 50-mL mark with solvent A. This will be the **internal standard spiking solution**.

JauWaterMix 4: 75 ng/mL of mixed internal standards

e.g. Add 2.5 mL of JauWaterMix 3 (3.0 µg/mL) to a 100 mL volumetric flask and dilute it to the mark with solvent B. (This is used as dilution solvent when the concentration of analytes is higher than the maximum point in the calibration curve)

3.5.2 Sample Extraction

The JAU 6476 sample is sensitive to sunlight. Keep the water sample away from light and store it in an amber bottle.

Figure 1 shows the analytical scheme for the extraction of JAU 6476 and its metabolites from water. The detailed stepwise procedure is summarized as follows:

Step 1. Measure 50-mL aliquots of water samples into the 50 or 100-mL graduated cylinders.
(If fortification is needed, please refer to Section 3.5.6)

Step 2. Add 100-µL of the 3.0 µg/mL mixed internal standard solution (JauWaterMix 3) and shake to mix the solutions. This is equivalent to a nominal 6-ppb solution based on a 50 mL sample size and concentrate to 4.0 mL final volume. (The final concentration of the internal standards in 4.0 mL is 75 ng/mL).

Step 3. Condition a 500 mg of C_{18} SPE cartridge (Varian, Part 1411-3027, 10 mL or Alltech Extract Clean Part 305250, 18 mL) or equivalent by rinsing it with about 10 mL acetonitrile and followed by 10-15 mL of HPLC-grade water. Do not allow the cartridge to go to dryness.

Step 4. Pass the 50 mL water sample through the cartridge at a rate of 10-20 mL/min.

Step 5. Rinse the graduated cylinder with about 5 mL of HPLC grade water. Add the rinsing water back to the cartridge. Let the water drain through the cartridge. Purge the cartridge with nitrogen and dry the cartridge under vacuum for 1-2 minutes.

Step 6. Elute the sample from the cartridge with 4 mL of acetonitrile : water (9:1, v/v) and collect it in a 13-mL centrifuge tube.

Step 7. Transfer 2 mL of extract into a HPLC vial and store it in a freezer (< -7 °C) until ready for LC/ESI/MS/MS analysis.

Step 8. Store the remaining extract (2 mL) in another vial and keep it in a freezer for future use.

3.5.3 Calibration Standards

The concentration of the sample matrix is defined in terms of ppb, which is ng per mL of the original sample matrix. This definition takes into account that the final extract contains analytes from 50 mL of water in a 4.0 mL final volume.

The calibration curve is prepared with five solvent levels using 0.15, 0.3, 1.8, 6.0 and 18.0-ppb standards (concentration in sample equivalent for a 50-mL sample), each containing 6-ppb internal standards (concentration in sample equivalent for a 50-mL sample). The first set is run at the beginning of the analysis, and the last set is run at the end of the analysis. The concentration of internal standard is kept at 75 ng/mL which is equivalent to 6 ppb sample.

JauWaterCal 1 *1.875 ng/mL standard or 0.15 ppb sample equivalent*:
E.g Add 0.313 mL of the 0.3-µg/mL mixed native solution (JauWaterMix 2) and 1.25 mL of 3.0-µg/mL of mixed internal standards (JauWaterMix 3) into a 50-mL volumetric flask, bringing to volume with Solvent B.

JauWaterCal 2 *3.75 ng/mL standard or 0.3 ppb sample equivalent*:
E.g Add 0.625 mL of the 0.3-µg/mL mixed native solution (JauWaterMix 2) and 1.25 mL of 3.0-µg/mL of mixed internal standards (JauWaterMix 3) into a 50-mL volumetric flask, bringing to volume with Solvent B.

JauWaterCal 3 *22.5 ng/mL or 1.8 ppb sample equivalent:*
E.g Add 0.375 mL of the 3.0-µg/mL mixed native solution (JauWaterMix 1) and 1.25 mL of 3.0-µg/mL of mixed internal standards (JauWaterMix 3) into a 50-mL volumetric flask, bringing to volume with Solvent B.

JauWaterCal 4 *75.0 ng/mL standard or 6.0 ppb sample equivalent*:
E.g Add 1.25 mL of the 3.0-µg/mL mixed native solution (JauWaterMix 1) and 1.25 mL of 3.0-µg/mL of mixed internal standards (JauWaterMix 3) into a 50-mL volumetric flask, bringing to volume with Solvent B.

JauWaterCal 5 *225.0 ng/mL standard or 18 ppb sample equivalent*:
E.g Add 3.75 mL of the 3.0-µg/mL mixed native solution (JauWaterMix 1) and 1.25 mL of 3.0-µg/mL of mixed internal standards (JauWaterMix 3) into a 50-mL volumetric flask, bringing to volume with Solvent B.

3.5.4 LC-MS/MS Analysis

These conditions are suggested based on the instrument and model used in method development and validation. LC and/or MS conditions may be changed if deemed necessary to obtain acceptable chromatographic performance or MS sensitivity.

3.5.4.1 HPLC Conditions

ThermoFinnigan P-4000 quaternary pump with a ThermoFinnigan degasser and A3000 autosampler.

Column:	Luna C18(2), 100 x 4.6 mm, 5µm (Phenomenex, Part No. 00D-4252-E0)
Injection volume:	50 µL
Column temp:	40 °C (built-in column heater)
Flow rate:	800 µL/min
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile
Split ratio:	4:1 (i.e., 80% to waste and 20% to MS)

Gradient:

Time (min)	%B
0	45
5.0	95
7.5	95
7.6	45
9.0	45

Retention times:

Compound	Retention time (t_R)
JAU 6476-thiazocine (K-972)	3.5
Desthio (K-913)	4.8
JAU 6476 (K-879)	5.4
S-methyl (K-880)	6.7

3.5.4.2 MS Conditions

Instrument	ThermoFinnigan TSQ 7000 triple quadrupole
Interface:	Atmospheric pressure API II in electrospray ionization (ESI) mode
Scanning Mode:	Selected Reaction Monitoring (SRM)
Capillary temp:	320 °C
Spray Voltage:	4.5 kV
Sheath Gas:	Nitrogen 80-100 psi
Auxiliary Gas:	Nitrogen 10-20 mL/min
Collision Gas:	Argon at ~2.2-2.4 mtorr

Ion Transition (Selected Reaction Monitoring)

Compound	Parent Ion (amu / Mode)	Daughter Ion (amu)	Scan Time (s)	Collision Energy (eV)
JAU 6476	344 / +	326	0.4	-13
JAU 6476 (IS)	349 / +	331	0.4	-13
Desthio	312 / +	70	0.25	-25
Desthio (IS)	317 / +	75	0.25	-25
JAU 6476 thiazocine	308 / +	190	0.4	-28
JAU 6476 thiazocine (IS)	313 / +	195	0.4	-28
S-methyl	358 / +	116	0.5	-26
S-methyl (IS)	362 / +	120	0.5	-26

All daughter ions are monitored at 1.0 amu resolution (e.g. 69.5 to 70.5 for Desthio) except for JAU 6476. The JAU 6476 daughter ion is monitored at 0.4 amu resolution (i.e., 325.5 to 325.9) so as to minimize the interference from control matrices.

3.5.5 Quantitation of Analyte

Quantitation of the native analyte was based on duplicate, five level calibration curves with a concentration range from 0.15 to 18 ppb. The peak area ratio of native to internal standard of each compound was plotted with its standard concentration. Using linear equation (without forcing zero) with weighing factor of $1/x^2$, the amount of unknown in the samples can be obtained by the following equation:

$$\text{Standard Concentration (ppb)} = \left(\frac{\text{Native Area}}{\text{Internal Standard Area}} - \text{y-intercept}\right) \times \frac{1}{\text{slope}}$$

3.5.6 Method Validation

Recovery tests and validations are generally performed according to the particular study protocol. In general, standard JauWaterMix 2 (0.3 µg/mL) will be used as the native spiking solution: 50 µL (15 ng) of JauWaterMix 2 will be added to the control water (50 mL) to represent the 0.3 ppb fortification level, while 500 µL (150 ng) of JauWaterMix 2 will be added to the water (50 mL) to represent the 3 ppb fortification level. Other fortification levels may be used as necessary.

Measure 50-mL aliquots of water samples into the 50 or 100-mL graduated cylinder
(If fortification is needed, please refer to section 3.5.6)

•

Add 100-µL of the 3.0 µg/mL of mixed internal standard (JauWaterMix 3)•
Shake and Vortex for • 15 s

•

Condition a 500 mg of C18 SPE cartridge by rinsing it with 10 mL ACN
and followed by 10-15 mL of water

•

Pass the 50 mL water sample through the cartridge at 10-20 mL/min

•

Rinse the graduated cylinder with about 5 mL of HPLC grade water.
Add the rinsing water back to the cartridge.
Let the water drain through the cartridge

•

Dry the cartridge under vacuum for 1-2 min.

•

Elute the sample from the cartridge with 4 mL of acetonitrile : water (9:1, v/v)
and collect it in a 13-mL centrifuge tube

•

Transfer 2 mL of extract into a HPLC vial and store it in a freezer (< -7 °C)
until ready for LC-MS/MS analysis

•

Store the remaining extract (2 mL) in another vial
and keep it in a freezer for future use

Figure 1. Analytical scheme for the extraction of JAU 6476 and its metabolites from water