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

1.0 SUMMARY

An analytical method was developed to quantify JAU 6476, Desthio (SSX 0665), S-methyl and thiazocine in soil and sediment using high-performance liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS). The method was validated from soil obtained from Lyons, (New York) at 10 ppb. The JAU 6476, Desthio (SSX 0665), S-methyl and thiazocine 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 an hour. The extract was then centrifuged at about 2300 rpm (• 1127 g) for about 10 min. The centrifuged extracts (4 mL) were transferred to a culture tube and heavy isotope internal standards were added. An aliquot of the extract (700 µL) was added to a HPLC vial and diluted with 300 µL of water. The resultant solution was analyzed by LC-MS/MS, and quantitation was done against known amount of heavy 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)
- I-Chem vials, 60-mL (I-Chem S236-0060)
- Analytical Balance (Mettler A163)
- Balance, Top loader, capable of weighing to the nearest 0.01 g
- Mechanical reciprocating shaker (Eberbach No. 6010)

- Pipette (Eppendorf 013950)
- SP Vortex mixer (Baxter S8223-1)
- Centrifuge (Damon/IEC Model DPR-6000)
- 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 and gradient HPLC, or equivalent (Finnigan Corp)

3.2 Reagents and Solvents

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

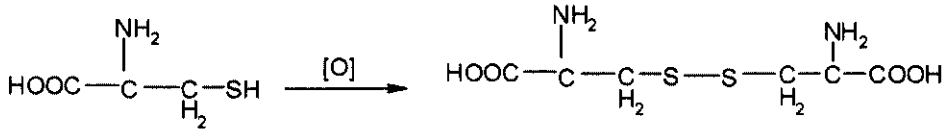
- Methanol (MeOH; HPLC Grade, Burdick & Jackson #230-4)
- Acetonitrile (ACN; HPLC Grade, Burdick & Jackson #015-4)
- Cysteine hydrochloride ($C_3H_7NO_2S.HCl.H_3O$, Fischer Scientific, #BP376-100, CAS 7048-04-6)
- Formic acid (88%, J.T. Baker)
- Water (Self-regenerated ion-exchange Millipore water)

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.

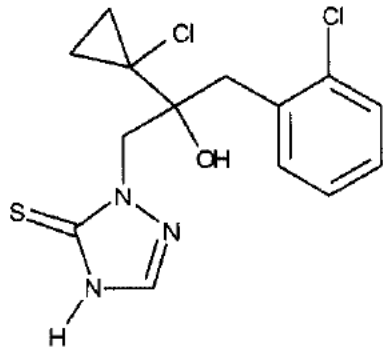
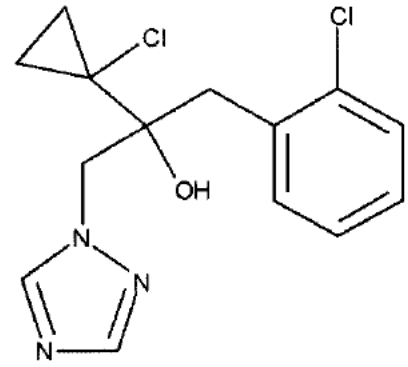
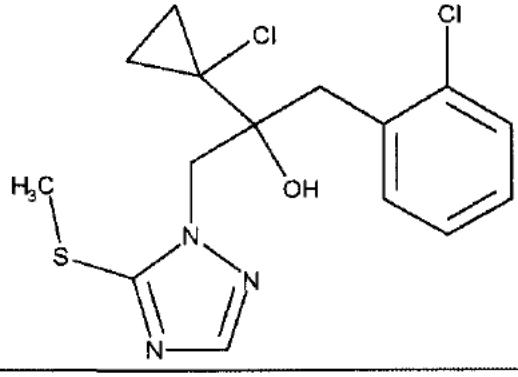
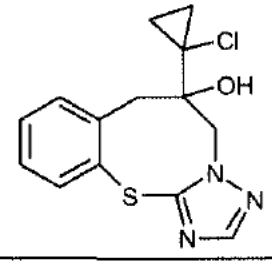
Solvent C: Acetonitrile / Water / Cysteine hydrochloride* (800/200/0.5, v/v/w)
Add 0.5 g cysteine hydrochloride into 200-mL water. Shake until it is dissolved.
Add 800-mL of acetonitrile into the solvent.

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.

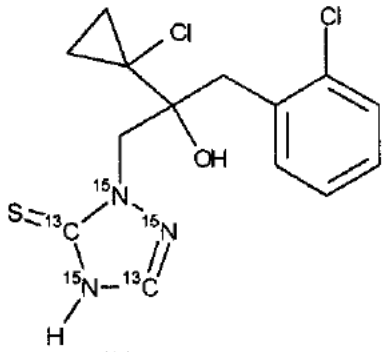
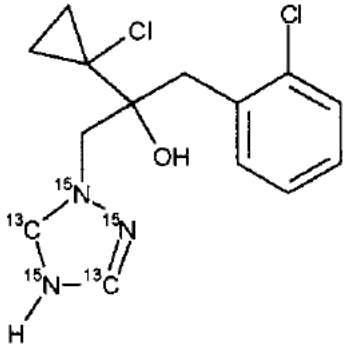
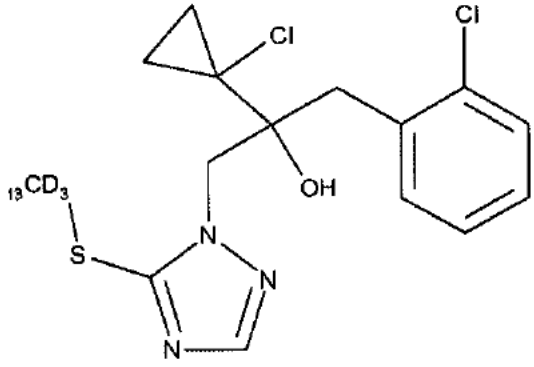
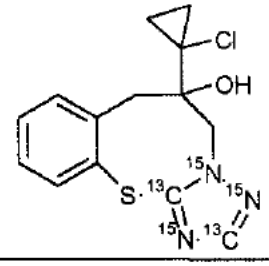


Cysteine Cystine

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-894 or equivalent $C_{12}{}^{13}C_2H_{15}Cl_2{}^{15}N_3OS$ 348.03 99.4% 01/03/2005	
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 "µg/g" (ppm) or "ng/g" (ppb) of original matrix 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 "ppm" 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. 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-894 (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 Mix 1-3 must be adjusted for the absolute concentration of the stock solutions to result in a near exact concentration for these solutions)

Mix 0: 0.1 µg/mL of mixed native standards

E.g. Add • 0.1 mL (• 10 µg) of each native standard (K-879, K-913, K-880 and K-972) to a 100-mL volumetric flask. Dilute to the 100-mL mark with solvent A. The final concentration is 0.1 µg/mL.

Mix 1: 1.0 µg/mL of mixed native standards
E.g. Add • 1 mL (• 100 µg) of each native standard (K-879, K-913, K-880 and K-972) to a 100-mL volumetric flask. Dilute to the 100-mL mark with solvent A. The final concentration is 1 µg/mL.

Mix 2: 10 µg/mL mixed native standards
E.g. Add • 10 mL (• 1000 µg) of each native standard (K-879, K-913, K-880 and K-972) to a 100-mL volumetric flask. Dilute to the 100-mL mark with solvent A. The final concentration is 10 µg/mL. This will be the **native spiking solution**.

Mix 3: 10 µg/mL mixed internal standards
E.g. Add • 10 mL (• 1000 µg) of each labeled internal standard (K-894, K-893, K-895 and K-988) to a 100-mL volumetric flask. Dilute to the 100-mL mark with solvent A. The final concentration is 10 µg/mL. This will be the **internal standard spiking solution**.

3.5.2 Sample Extraction

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

Step 1. Weigh about 15 g soil into a 60-mL I-Chem glass vial.
(If fortification is needed, please refer to Section 3.5.6)

Step 2. Add 30-mL of Solvent C (Acetonitrile / Water / Cysteine hydrochloride [800/200/0.5, v/v/w]) into the glass vial.

Step 3. With a mechanical shaker, shake for 1 h at • 130 cycles/min at ambient temperature.

Step 4. Remove the glass vial from the shaker and centrifuge at about 2300 rpm (• 1127 *g*) for about 10-15 min.

Step 5. Pipette 4 mL of the extract into disposable culture tube or equivalent. (*Aliquot factor = 4/30*)

Step 6. Add 40 µL of Mix 3 (Internal Standard) into the same culture tube. (IS in the culture tube = 100 ng/mL)

Step 7. Vortex the culture tube for • 15 s

Step 8. Pipette 700 µL of the extract from step 7 into a HPLC vial. Add 300 µL of water into the same vial. [Aliquot factor = (4/30 * 0.7/4) = 0.7 / 30]. Cap and vortex for ≈ 15 s.

Step 9. Ready for LC/MS/MS analysis. (Keep the remaining extract from step 7 in a freezer)

3.5.3 Calibration Standards

The concentration of the sample matrix is defined in terms of ppb, which is ng per g of the original sample matrix. This definition takes into account that the final extract contains analytes from 0.35 g soil in a 1 mL final volume. (Fifteen grams soil extracted with 30 mL solvent and 0.7 mL of extract was pipetted into a HPLC vial and diluted to 1.0 mL final volume)

The calibration curve is prepared with five levels in duplicate. The first set is run at the beginning of the analysis and the second is run at the end of the analysis.

Mix 4: 200 ppb sample equivalent (i.e. 70 ng/mL)
E.g. Add 0.35 mL Mix 2 and 0.35 mL Mix 3 to a 50-mL volumetric flask. Dilute to 50 mL with solvent B. (IS = 70 ng/mL).

Mix 5: 100 ppb sample equivalent (i.e. 35 ng/mL)
E.g. Add 0.175 mL Mix 2 and 0.35 mL Mix 3 to a 50-mL volumetric flask. Dilute to 50 mL with solvent B.

Mix 6: 50 ppb sample equivalent (i.e.17.5 ng/mL)
E.g. Add 0.875 mL Mix 1 and 0.35 mL Mix 3 to a 50-mL volumetric flask. Dilute to 50 mL with solvent B.

Mix 7: 10 ppb sample equivalent (i.e. 3.5 ng/mL)
E.g. Add 0.175 mL Mix 1 and 0.35 mL Mix 3 to a 50-mL volumetric flask. Dilute to 50 mL with solvent B.

Mix 8: 5 ppb sample equivalent (i.e. 1.75 ng/mL)
E.g. Add 87.5 µL Mix 1 and 0.35 mL Mix 3 to a 50-mL volumetric flask. Dilute to 50 mL with solvent B.

3.5.4 LC-MS/MS Analysis

These conditions are suggested based on the instrument and model used. 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:	75 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:	325 °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 5 to 200 ppb. The peak area ratio of native to internal standard of each compound was plotted with its standard concentration. Using equal weigh linear regression and forcing the curve to go through origin, the amount of unknown can be obtained by the following equation:

$$\text{Concentration (ppb)} = \frac{\text{Native Area}}{\text{Slope x Internal Standard Area}}$$

3.5.6 Method Validation

Recovery tests and validations are generally performed according to the particular study protocol. In general, standard Mix 1 and Mix 2 will be used as the native spiking solution: 150 µL (150 ng) of Mix 1 will be added to the soil (15 g) to represent the 10 ppb fortification level, while 150 µL (1500 ng) of Mix 2 will be added to the soil (15 g) to represent the 100 ppb fortification level. Other fortification levels may be used as necessary.

Weigh 15 g soil into 60 mL I-Chem vial
(If fortification is needed, please refer to section 3.5.6)

●

Add 30-mL of extraction solvent [ACN/water/Cysteine hydrochloride (800/200/0.5, v/v/w]

●

Shake in a mechanical shaker for 1 hour

●

Centrifuge it at about 2300 rpm (• 1127 *g*) for about 10-15 min

●

Pipet 4 mL of the extract into disposable culture tube

●

Add 40 µL of 10 µg/mL of mixed internal standard

●

Vortex the culture tube for • 15 s.

●

Pipet 700 µL of the extract into HPLC vial

●

Add 300 µL of water into the same HPLC vial

●

Cap and vortex for • 15 s

●

Store these in a freezer until ready for LC-MS/MS

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