1.0 INTRODUCTION

Methodology was validated to determine the content of S-2188 in freshwater (reconstituted for hardness). The analytical method was validated with regards to accuracy and precision, linearity, specificity, limit of detection and limit of quantitation. The method was validated by fortification of freshwater (reconstituted for hardness) with S-2188 at concentrations of 1.00 (LOQ), 500 and 20,000 µg a.i./L. Recovery samples containing the test substance were concentrated using solid phase extraction (SPE) and analyzed by liquid chromatography/mass spectrometry (LC/MS/MS).

The study was initiated on 19 January 2006, the day the Study Director signed the protocol, and was completed on the day the Study Director signed the final report. The experimental portion of the study was conducted on 19 January 2006 at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. All original raw data, the original protocol, and the original final report produced during this study are archived at Springborn Smithers Laboratories at the above location. The stored data will be discarded after a written agreement with the Sponsor.

2.0 MATERIALS AND METHODS

2.1 Study Protocol

Procedures used during this method validation study followed those described in the Springborn Smithers protocol entitled "Validation of the Analytical Method for the Determination of a Test Substance in Aqueous Solutions Following OPPTS 860.1340, SANCO/3029/99 rev.4 and SANCO/825/00 rev.7," Springborn Smithers Protocol No.: 012505/Method Validation/SANCO/S-2188.

2.2 Test and Reference Substances

2.2.1 Test Substance

The test substance, S-2188, was received on 18 October 2005 from Sumitomo Chemical Company, Ltd., Tokyo, Japan. The following information was provided:

Name:	S-2188 technical grade
Synonym:	pyrazoline fungicide
Lot No.:	030-050914-1G
CAS No.:	Not listed
Purity:	94.7% (Appendix 2)
Recertification Date:	5 October 2008

Upon receipt at Springborn Smithers, the test substance (SSL No. 114-72) was stored at room temperature in the original container in a dark ventilated cabinet. The test substance was moved to refrigerated storage (1 to 10 °C) on 21 October 2005 at the request of the Study Sponsor. Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

2.2.2 Reference Substance

The reference substance, S-2188, was received on 18 October 2005 from Sumitomo Chemical Company, Ltd., Tokyo, Japan. The following information was provided:

Name:	S-2188 analytical standard
Lot No.:	4CM03-R2G
CAS No.:	Not listed
Purity:	99.4% (Appendix 2)
Recertification Date:	7 July 2008

Upon receipt at Springborn Smithers, the reference substance (SSL No. 114-73) was stored at room temperature in the original container in a dark ventilated cabinet. The reference substance was moved to refrigerated storage (1 to 10 °C) on 21 October 2005 at the request of the Study Sponsor. Concentrations were adjusted for the purity of the reference substance and are presented as active ingredient (a.i.).

Determination of stability and characterization, verification of the test and reference substance identity, maintenance of records on the test and reference substances, and archival of a sample of the test and reference substance are the responsibility of the Study Sponsor.

2.3 Reagents

1.	Acetonitrile:	Burdick & Jackson, reagent grade
2.	Acetic acid:	VWR, reagent grade
3.	Reagent grade water:	Prepared from a Sybron/Barnstead NANOpure® water purification system (meets ASTM Type II requirements)

2.4 Test System

The freshwater (reconstituted for hardness) used during this study was prepared by adding the following quantity of salts required (147 mg $NaHCO_3$/L, 92 mg $CaSO_4 \cdot H_2O$/L, 92 mg $MgSO_4$/L and 6.0 mg KCl/L) to laboratory well water to produce a pH of 8.1, hardness and alkalinity (as $CaCO_3$) of 160 mg/L and 110 mg/L, respectively, and a specific conductance of 500 µmhos/cm. In addition, representative samples of the freshwater source were analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration of the water source was 1.5 mg/L for January 2006.

2.5 Equipment

1.	Instrument:	MicroMass Quattro Micro mass spectrometer equipped with MicroMass Electro Spray, a Waters Model 2695 quaternary pump, a Waters Model 2695 autosampler, a Waters Model 2695 membrane degasser, a Waters Model 2695 column oven and MassLynx software version 4.0 for data acquisition
2.	Balance:	American Scientific Products Model SP 182
3.	Laboratory equipment:	Syringes, volumetric flasks, volumetric pipets, Pasteur pipets, test tubes, autosampler vials and glass amber bottles, Teflon®-lined caps
4.	SPE column:	Waters Oasis HLB, 500 mg, 6.0 mL

2.6 Preparation of Stock Solutions

A 20.0 mg a.i./mL primary test substance stock solution was prepared by placing 0.5289 g of the test substance (0.5009 g as active ingredient) in a 25.0-mL volumetric flask and bringing it to volume with acetonitrile. This stock solution was used to fortify the high concentration recovery samples. A secondary stock solution (200 mg a.i./L) was prepared by placing 0.500 mL of the 20.0 mg a.i./mL primary stock solution in a 50.0-mL volumetric flask and bringing it to volume with acetonitrile. A further dilution (1.00 mg a.i./L) was prepared by placing 0.500 mL of the 200 mg a.i./L secondary stock solution in a 100-mL volumetric flask and bringing it to volume with acetonitrile. A 1000 mg a.i./L stock solution was prepared by placing 5.00 mL of the 20 mg a.i./mL primary stock solution in a 100-mL volumetric flask and bringing it to volume with acetonitrile. The 1.00 and 1000 mg a.i./L stock solutions were used to fortify the LOQ and low concentration recovery samples, respectively.

A 1.00 mg a.i./mL primary reference substance stock solution was prepared by placing 0.1008 g of the reference substance (0.1002 g as active ingredient) in a 100-mL volumetric flask and bringing it to volume with acetonitrile. A 1.00 mg a.i./L secondary stock solution was prepared by placing 0.100 mL of the primary stock solution in a 100-mL volumetric flask and bringing it to volume with acetonitrile. This stock solution (1.00 mg a.i./L) was used to prepare calibration standards.

All stock solutions were stored in a refrigerator (4 °C) in glass amber bottles fitted with Teflon®-lined caps.

2.7 Preparation of Calibration Standards

The calibration standards were prepared in 1000:1000:0.5 acetonitrile:purified reagent water:acetic acid using the 1.00 mg a.i./L stock solution to yield concentrations of 1.00, 5.00, 10.0, 25.0 and 50.0 µg a.i./L.

2.8 Recovery Sample Fortification

The recovery samples were prepared at concentrations of 1.00 (LOQ), 500 and 20,000 µg a.i./L by fortifying aliquots of stock solution with freshwater (reconstituted for hardness). The recovery samples were prepared as described below:

Sample	Stock Solution Used	Volume of Stock Used (mL)	Sample Volume (mL)	Recovery Sample Concentration (µg a.i./L)
LOQ	1.00 mg a.i./L	0.100	100	1.00
LOW	1000 mg a.i./L	0.050	100	500
HIGH	20.0 mg a.i./mL	0.100	100	20,000

The high concentration samples (20,000 µg a.i./L) did not immediately go into solution and were sonicated for approximately 30 minutes. Following sonication, these samples were observed to be homogeneous solutions. Five replicates were prepared for each concentration level. In addition, five recovery samples were left unfortified to serve as controls.

2.9 Solid Phase Extraction

Recovery samples were further concentrated by an SPE technique using solid phase extraction columns (Waters Oasis HLB, 6.0 mL, 500 mg column). Each SPE column was conditioned with one column volume of methanol (6 mL) followed by one column volume of purified reagent water (6 mL). The recovery samples were then loaded on the columns and eluted at moderate flow (using 7 psi of vacuum). The SPE columns were dried at a vacuum pressure of 20 psi for 10 minutes, and eluted with 5.00 mL of acetonitrile. The eluate was then diluted with 1000:0.5 purified reagent water:acetic acid so that the minimum final volume was 10.0 mL for the final extracts. Further dilution was made in 1000:1000:0.5 acetonitrile:purified reagent water:acetic acid, if necessary, to ensure that the analyte concentrations were within the calibration range. The prepared samples were transferred into amber autosampler vials and analyzed by liquid chromatography/mass spectrometry (LC/MS/MS). Each recovery sample was injected singly.

2.10 Evaluation of Precision, Accuracy, Specificity and Linearity

Accuracy and precision were evaluated in terms of the mean percent recovery, standard deviation (SD) and percent standard deviation (RSD) of the recovery samples based on the integrated peak area. Specificity of the method was determined by examination of the control samples for peaks at the same retention time as S-2188 which might interfere with the quantitation of S-2188. Linearity of the method was determined by the coefficient of determination (r^2).

2.11 Limit of Quantitation and Detection

Five replicate samples of control matrix were fortified at the limit of quantitation (LOQ, 1.00 µg a.i./L) for the analysis. The precision of the analytical method at the LOQ was calculated by determining the RSD of the percent recoveries. The limit of detection (LOD) was calculated as described in Section 3.0.

2.12 Analysis

An aliquot was removed from each replicate recovery sample for analysis by liquid chromatography/mass spectrometry (LC/MS/MS). The LC/MS/MS analysis was conducted utilizing the following instrumental conditions:

HPLC parameters:

Column:	YMC ODS-AQ, S-3, 2.0 x 50 mm, 3-µm, 120 Å
Mobile phase:	0.05% acetic acid in 50:50 acetonitrile:purified reagent water
Flow rate:	200 µL/minute
Injection volume:	10.0 µL
Temperature:	20 °C
Retention time of S-2188:	approximately 4.3 minutes
MS parameters:	
Instrument:	MicroMass Quattro Micro Mass Spectrometer
Interface:	MicroMass Electro Spray
Ionization mode:	positive
Dwell time:	0.75 seconds
Desolvation temperature:	325 °C
Q1/Q3 mass:	332.0/231.0 amu

2.13 Preparation of Standard Curve

Two sets of calibration standards were analyzed with each recovery sample set; one set prior to analysis of the recovery samples, and the second set immediately following the analysis of the recovery samples. Injection of samples and calibration standards onto the LC/MS/MS system was performed by programmed automated injection. A single injection was performed for each standard solution.

3.0 CALCULATIONS

A calibration curve was constructed by plotting the analyte concentration (µg a.i./L) of the calibration standards against the peak area of the analyte in the calibration standards. The two sets of calibration standards analyzed before and after each recovery sample set were plotted in a single calibration curve, and the calibration curve was used for calculation of the concentration of test substance in the recovery sample set. The equation of the line (equation 1) was algebraically manipulated to give equation 2. The concentration of test substance in each recovery sample was calculated using the slope and intercept from the linear regression analysis, the detector response, and the dilution factor of the recovery sample. Equations 2 and 3 were then used to calculate measured concentrations and analytical results.

(1) $y = mx + b$

(2) $DC(x) = \frac{(y - b)}{m}$

(3) $A = DC \times DF$

where:

x	=	analyte concentration
y	=	detector response (LC/MS/MS peak area output) from the chromatogram
b	=	y-intercept from the regression analysis
m	=	slope from the regression analysis
DC (x)	=	detected concentration (µg a.i./L) in the sample
DF	=	dilution factor (final volume of the sample extracts divided by the original water sample volume, minimum DF was 0.100 (10 mL/100 mL))
A	=	analytical result (µg a.i./L), concentration in the original sample

The limit of detection (LOD) was calculated using the following equations:

$$LOD_{INST} = C_{LS}$$

$$LOD = LOD_{INST} \times DF_{CNTL}$$

where:

C_{LS}	=	Concentration of the lowest calibration standard, at which peak was detected with S/N greater than three
LOD_{INST}	=	Limit of detection at the instrument where adequate peak was obtained (1.00 µg a.i./L)
DF_{CNTL}	=	Dilution factor of the control samples (smallest dilution factor used, 0.100)
LOD	=	Limit of detection in test system for this analytical method (0.10 µg a.i./L in aqueous solution)