1.0 INTRODUCTION

Methodology was validated to determine the residue levels of metconazole (*cis*- and *trans*-isomers) in seawater. The analytical method was validated following the EPA Ecological Effects Test Guidelines, OPPTS 850.7100 (draft), 1996. The detailed Sponsor-supplied method is presented in Appendix 1. In general, 3.0 g of sodium chloride was added to all recovery samples (100 mL), which contained *cis*- and *trans-metconazole*. The recovery samples were extracted using 50.0 mL of a 4:1 (v:v) hexane/ethyl acetate solution, reconstituted in toluene and analyzed by automated injection using gas chromatography with nitrogen phosphorous detection (GC/NPD).

The study was initiated on 13 December 2004, 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 ILV study was conducted from 22 December 2004 to 11 January 2005 at Springborn Smithers Laboratories (SSL), located in Wareham, Massachusetts. All original raw data and the original final report produced during this study are archived at Springborn Smithers Laboratories at the above location.

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 "Metconazole (KNF-S-474m): Independent Laboratory Validation (ILV) - Determination of Metconazole Residues in Water," Springborn Smithers Laboratories Protocol No.: 092104/OPPTS-ILV/General.

2.2 Test Substances

2.2.1 *Cis-metconazole*

The test substance, *cis-metconazole*, was received on 28 September 2004 from Valent Technical Center, Dublin, California. The following information was provided:

Name:	*cis-metconazole*
Lot No.:	AS-2025a
CAS No.:	125116-26-6 (metconazole)
Purity:	99.3%
Recertification Date:	7 February 2007

Upon receipt at Springborn Smithers, the test substance (SSL No. 108-34) was stored under nitrogen in a freezer (< -4 °C). Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

2.2.2 *Trans-metconazole*

The test substance, *trans-metconazole*, was received on 28 September 2004 from Valent Technical Center, Dublin, California. The following information was provided:

Name:	*trans-metconazole*
Lot No.:	AS-2026a
CAS No.:	125116-26-6 (metconazole)
Purity:	99.3%
Recertification Date:	8 February 2007

Upon receipt at Springborn Smithers, the test substance (SSL No. 108-35) was stored under nitrogen in a freezer (< -4 °C). Concentrations were adjusted for the purity of the test substance and are presented as active ingredient (a.i.).

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

2.3 Reagents

1.	Acetone:	Burdick & Jackson, reagent grade
2.	Toluene:	Burdick & Jackson, reagent grade
3.	Hexane:	Burdick & Jackson, reagent grade
4.	Ethyl acetate:	Burdick & Jackson, reagent grade
5.	Sodium chloride:	Fisher, reagent grade

2.4 Equipment

1.	Instrument:	Hewlett Packard model 6890 Series gas chromatograph equipped with a nitrogen phosphorous detector, Hewlett Packard autosampler and injector Series 7683, and a Hewlett Packard ChemStation Version A.06.03.
2.	Balance:	Mettler AE200, Mettler PJ3000
3.	Laboratory equipment:	Syringes, volumetric pipets, Pasteur disposable pipets, volumetric flasks, separatory funnels, round bottom flasks, rotary evaporator, screw-cap glass vials, autosampler vials, amber bottles, and graduated cylinders

2.5 Preparation of Stock Solutions

A 1.00 mg a.i./mL primary stock solution was prepared for both *cis-* and *trans-metconazole* by weighing 0.0253g of each test substance (0.0251 g as active ingredient) into separate 25-mL volumetric flasks and bringing each to volume with acetone.

A 0.5-mL aliquot of each primary stock solution (1.00 mg a.i./mL) was combined in a 50.0-mL volumetric flask and brought to volume with acetone. The final stock solution had a concentration of 10.0 μg a.i./mL for both *cis-* and *trans-* isomers in acetone. An additional 0.5-mL aliquot of each primary stock solution (1.00 mg a.i./mL) was combined in another 50.0-mL volumetric flask and brought to volume with toluene. The final stock solution had a concentration of 10.0 μg a.i./mL in toluene. The acetone stock was used to fortify recovery samples and the toluene stock was used to fortify calibration standards.

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

2.6 Preparation of Calibration (Linearity) Standards

Calibration standards werc prepared in toluene at concentrations of 0.0250, 0.0500, 0.200, 0.500 (continuing standard) and 1.00 μg a.i./mL using the 10.0 μg a.i./mL toluene stock solution.

2.7 Recovery Sample Fortification

The *cis-* and *trans-metconazole* recovery samples were prepared at concentrations of approximately 0.00500 (LOQ) and 0.0500 μg a.i./mL by fortifying the appropriate volume of the 10.0 μg a.i./mL stock solution (in acetone) into 100 mL of seawater. Five replicates were prepared for the 0.00500 (LOQ) and 0.0500 μg a.i./mL treatment levels. In addition, two recovery samples were left unfortified to serve as controls, and one reagent blank containing only seawater was also prepared.

2.8 Trial #1 Extraction Procedure

In order to decrease headspace and reduce losses due to volatility, 250-mL separatory funnels were used to prepare recovery samples. *Cis-* and *trans-metconazole* samples were prepared as described above and extracted once using 10.0 mL of a 4:1 (v:v) hexane/ethyl acetate solution. Samples were hand-shaken for one minute and layers were allowed to separate, then 1.00 mL of the organic layer was added to a 30-mL screw cap glass vial. The extracts were reduced to dryness by rotary evaporation at <40 °C, reconstituted with 1.00 mL toluene, vortexed for 30 seconds, and then sonicated for five minutes. The extracts were transferred into amber autosampler vials and analyzed by automated injection using gas chromatography with nitrogen phosphorous detection (GC/NPD).

2.9 Analysis

The GC/NPD analysis was conducted utilizing the following instrumental conditions:

Column:	Agilent DB-5, 30.0 m x 0.53 mm x 1.5 µm	
Gas flows:	Carrier gas -	Helium held at a constant flow of 3 mL/minute
	Detector gas -	Hydrogen at 3 mL/minute, Air at 60 mL/minute, Helium make-up gas at a constant combined flow of 10 mL/minute
Temperatures:	Injector at 280 °C Detector at 310 °C	
Oven:	Initial temp at 235 °C for 11 minutes Ramp at 25 °C/minute to 285 °C hold for 2.00 minutes	
Injection Volume:	3 µL; splitless (purge @ 1.0 minutes)	
Retention Time:	approximately 11.1 minutes – *cis-metconazole* approximately 11.9 minutes – *trans-metconazole*	

The first trial (Trial #1) failed A second extraction method was attempted, increasing the amounts of extraction solvent to 50 mL and adding sodium chloride to the samples

2.10 Trial #2 Extraction Procedure

In order to decrease headspace and reduce losses due to volatility, 250-mL separatory funnels were used to prepare recovery samples. *Cis-* and *trans-metconazole* samples were prepared as described above and 3.0 g of sodium chloride was added to each sample. The recovery samples were extracted using 50.0 mL of a 4:1 (v:v) hexane/ethyl acetate solution. Samples were hand-shaken for one minute and layers were allowed to separate, then 5.00 mL of the organic layer was added to a 30-mL screw cap glass vial. The extracts were reduced to dryness by rotary evaporation at <40 °C, reconstituted with 1.00 mL toluene, vortexed for 30 seconds, and then sonicated for five minutes. The extracts were transferred into amber autosampler vials and analyzed by automated injection using the conditions described above in Section 2.9.

2.11 Linearity and Response Factor (RF)

The linearity standards were analyzed with each sample set to establish the linearity of the instrument. To verify the linearity of the response, response factors (RF) for each isomer for each standard were calculated by dividing the peak area by the standard concentration, and determining the coefficient of variation (% CV: standard deviation of the response factors/average response factor x 100) for the standard set.

The sample sequence was analyzed beginning and ending with the continuing standard (0.500 µg a.i./mL each isomer) and included a standard injection after every one to two sample injections. The RF for each isomer was calculated from each continuing standard injection in the sequence. The criteria for an acceptable sequence was a % CV $\leq$ 10% for all continuing standards and for the linearity standards.

2.12 Calculations

Residues of *cis-* and *trans-metconazole* were calculated from the average response factor (RF) for the continuing standards injected with the sample sequence. The individual response factors (*cis-* and *trans-* isomers) for each standard injection were determined by dividing the analyte response by the analytical concentration (area response / µg a.i./mL).

The analyte concentration in the sample extract (µg a.i./mL) was calculated using the following equation:

$$\text{analyte concentration } (cis \text{ or } trans) = \frac{\text{sample response}}{\text{average RF}} \times \text{DF}$$

The total metconazole in the sample (µg a.i./mL) was calculated using the following equation:

$$\text{total metconazole} = cis \text{ analyte concentration} + trans \text{ analyte concentration}$$

VALENT U.S.A. CORPORATION
VALENT TECHNICAL CENTER
DUBLIN, CALIFORNIA

DETERMINATION OF METCONAZOLE RESIDUES IN WATER

METHOD RM-41-W1 **DATE: September 13, 2004**

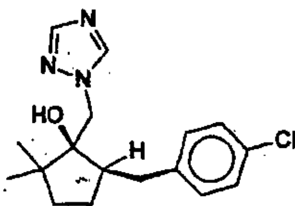
INTRODUCTION

This method determines residues of metconazole, (1*RS*, 5*RS* : 1*RS*, 5*SR*)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol [*cis* isomer: (1*RS*, 5*RS*), *trans* isomer: (1*RS*, 5*SR*)] in water samples.

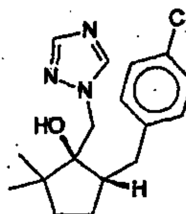
Briefly, metconazole residues are extracted from water using a hexane/ethyl acetate (4/1, v/v) mixture, in a 10:1 water:organic solvent ratio. A sample of the organic solvent is taken and analyzed by gas chromatography with a nitrogen-phosphorus flame-ionization detector (GC/NPD).

REFERENCE STANDARDS

Metconazole - CAS Number 125116-26-6



cis-metconazole



trans-metconazole

Stock Standard Solutions:

Prepare individual stock solutions of each isomer of metconazole (*cis* and *trans*), in acetone, containing 1.0 mg/mL (i. e. 0.10 g metconazole / 100 mL acetone). To ensure a 1.0 mg/mL concentration, correct the amount of standard weighed for the purity of the standard.

Fortifying Solutions:

Prepare a 0.01 mg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) fortifying standard in acetone. For example, combine 1.0 mL of the 1.0 mg/mL *cis*- metconazole stock solution with 1.0 mL of the 1.0 mg/mL *trans*- metconazole stock solution and dilute to 100 mL in acetone.

Analytical Standard Solutions:

Prepare a 10.0 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) standard in toluene. For example, mix 1.0 mL of each of the 1.0 mg/mL stock solution standards dilute to 100 mL in toluene. This solution will be used for making dilutions for analytical standards.

Prepare a 1.0 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) solution in toluene from the 10.0 µg/mL solution (i. e. 10.0 mL x 10 µg/mL standard / 100 mL toluene). This solution will be used as an analytical (linearity) standard.

Prepare a 0.50 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) solution in toluene from the 10.0 µg/mL solution (i. e. 5.0 mL x 10 µg/mL standard / 100 mL toluene). This solution will be used as an analytical (continuing and linearity) standard.

Prepare a 0.20 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) solution in toluene from the 10.0 µg/mL solution (i. e. 2.0 mL x 10 µg/mL standard / 100 mL toluene). This solution will be used as an analytical (linearity) standard.

Prepare a 0.05 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) solution in toluene from the 10.0 µg/mL solution (i. e. 0.50 mL x 10 µg/mL standard / 100 mL toluene). This solution will be used as an analytical (linearity) standard.

Prepare a 0.025 µg/mL (each *cis* and *trans* isomer, 1/1 *cis/trans*) solution in toluene from the 10.0 µg/mL solution (i. e. 0.25 mL x 10 µg/mL standard / 100 mL toluene). This solution will be used as an analytical (linearity) standard.

Other concentrations may be made as necessary. All standard solutions should be kept refrigerated when not in use.

REAGENTS

Ethyl Acetate – pesticide quality or equivalent
Hexane - pesticide quality or equivalent
Toluene - pesticide quality or equivalent.

REAGENT SOLUTIONS

Hexane/Ethyl Acetate, 4/1, v/v
Combine 4 parts hexane with 1 part ethyl acetate. For example, add 120 mL of hexane and 30 mL of ethyl acetate to a reagent bottle. Store at room temperature.

EQUIPMENT

Gas chromatograph:
Hewlett-Packard Model 5890, equipped with a packed column glass insert for direct injection (HP Part # 5080-8732, packed with approximately 5 mm of silanized glass wool), a Nitrogen Phosphorous detector (NPD), automatic sampler, and HP ChemStation or equivalent system.
Gas chromatographic columns:
DB-5, 30 M x 0.53 mm x 1.5 μm film
Pipettes, 10.0 mL volumetric.
Pipetor, Automatic, capable of accurately dispensing 0.01 to 2.5 mL volumes
Rotary Vacuum Evaporators with temperature controlled water baths (<40°C)
Rotary Evaporator Adapters: 24/40 glass joint and 13-425 screw thread, Wheaton #39633
Screw-thread 13-425 to 24-400, Sigma-Aldrich #Z106208
Separatory Funnels, 250 mL
Vials, 25 mL screw-capped 24-400 size

ANALYTICAL PROCEDURE

1. Extraction

If required by testing facility, control samples for method recovery should be fortified with metconazole (See Note 1) prior to adding solvent for the initial extraction. For the fortification samples, measure 100 mL of untreated water samples into 250 mL separatory funnels and spike over a range of 0.01 to 0.40 μg/mL metconazole (total *cis* + *trans*) / sample.

For sample analyses, accurately measure 100 mL of the samples into 250 mL separatory funnels. Add 10.0 mL of hexane/ethyl acetate (4/1, v/v) to the sample and shake for 1 minute. Allow the phases to separate, and transfer a 1.0 mL portion of the top, organic layer to a 25 mL vial (the lower aqueous phase may be drained off to facilitate sampling the organic phase). Evaporate the sample to dryness using rotary evaporation with a water temperature bath of <40°C. Redissolve the sample in 1.0 mL of toluene for GC analysis. (See Note 2).

2. Gas Chromatography Measurement

Analyze the samples by gas chromatography, using a nitrogen-phosphorus detector and the following instrument conditions:

Column:	DB 5 (30 M x 0.53 mm, 1.5 µm film), or equivalent.
Column temperature program:	
Initial Temp.:	235°C
Initial Time:	11 minutes
Rate:	25°C/minute
Final Temp.:	285°C
Final Time:	2 minutes
Run time:	15 minute
Injector temperature:	280°C
Detector temperature:	285°C
Carrier Gas and flow rate:	Helium, 10 mL/min (13.5 psi @ 235°C).
Makeup Gas and flow rate:	Helium, 20 mL/min.
Hydrogen flow rate:	3.6 mL/minute
Air flow rate:	105 mL/minute
Injection volume:	2.0 µl
Retention times:	9.1 minutes (*cis*-metconazole) 10.2 minutes (*trans*-metconazole)

The above parameters are given as an example. The parameters may be adjusted as necessary, but must provide adequate separation of the *cis* and *trans* isomers of metconazole.

Analyze a range of linearity standards with each analytical set to establish the linearity of the instrument. To verify the linearity of the response, calculate the response factors for each isomer for each standard by dividing the peak area (or height) by the standard concentration, and determine the coefficient of variation (%CV: standard deviation of the response factors/average response factor X 100). To be considered linear for the range of standards, the %CVs must be ≤ 10% for GC/NPD analyses.

Analyze the samples using a sequence that begins and ends with a continuing standard (0.5 µg/mL each isomer) and includes a standard injection after every one to three sample injections. Calculate the Response Factor for each isomer from each continuing standard injection in the sequence. For a sequence to be acceptable, the %CV for the continuing standards must be ≤ 10%.

The responses for all samples analyzed must be less than the response for the largest linearity standard. If any sample is larger than the largest standard, dilute the sample as appropriate, so it falls within the established linear range.

3. Calculations

Residues of *cis* and *trans* metconazole are calculated from an average response factor for the continuing standards injected with the sample sequence. The individual response factors (*cis* and *trans* isomers) for each standard injection are determined by dividing the analyte response by the analyte concentration (area response / µg/mL). The analyte concentration in the sample extract is calculated using the formula:

Analyte Conc. (*cis* or *trans* µg/mL) = sample response / Average Response Factor

and the total metconazole in the sample (µg/mL) is calculated as:

µg/mL total metconazole = *cis* Analyte Conc. + *trans* Analyte Conc. ÷ (SV ÷ EV) * DF

Where: SV = Sample volume (100 mL)
EV = Extract volume (10 mL)
DF = Dilution Factor (if samples are diluted for analysis)

LIMITS OF QUANTITATION AND DETECTION

The validated Limit Of Quantitation (LOQ) of metconazole in water samples analyzed by this method was 0.005 µg/mL for each isomer of metconazole. The estimated Limit Of Detection (LOD) is 0.0025 µg/mL for each isomer of metconazole. The method has been validated with fortifications ranging from 0.005 µg/mL (LOQ) to 0.20 µg/mL for each isomer of metconazole.

ANALYSIS TIME

A trained analyst, familiar with this method, can complete the sample work up for a set of ten to twelve samples in approximately one hour. The results are available within 24 hours of initiating the analysis.

CONFIRMATION OF RESIDUES

Sample residues may be confirmed by analyzing the samples using gas chromatography coupled with a mass selective detector using a positive chemical ionization mode (GC/MSD/PCI). GC, MS and PCI conditions, described below, are given as an example, and may be modified as necessary to achieve the sensitivity and selectivity necessary for the analyses. Quantitation of residues by GC/MSD/PCI is not as reliable as quantitation by the GC/NPD system described previously, however the GC/MSD/PCI may be used to confirm the presence or absences of metconazole residues in the samples.

Instrument: Agilent 6890 GC/6980 MSD system equipped with a chemical ionization source, split/splitless injector, and Chemstation software

GC Parameters:

Column: DB1701 (30 M x 0.25 mm, 0.25 µm film), or equivalent.

Column temperature program:

Initial Temp.:	160°C
Initial Time:	0 minutes
Rate:	25°C/minute
Temp. 2:	285°C
Time 2:	10 minute
Run time:	15 minute
GC/MS Interface temp.:	280°C
Carrier Gas and flow rate:	Helium, 1.5 mL/min., constant flow mode
Injection:	Splitless
Injector temperature:	275°C
Purge Flow & Time:	15 mL/min, on at 0.70 minutes
Injection volume:	2.0 µl

MS Parameters:

Source:	Positive Chemical Ionization
Reagent Gas:	Methane @ 20 psi
Source Temp:	250°C
Quad Temp:	150°C
SIM Parameters:	
Masses:	320.0, 348.0, 302.0 m/z
Dwell Time:	80 msec each
EM Voltage:	tune value + ~500
Solvent Delay:	3 minutes
Retention times:	7.0 minutes (*cis*-metconazole)
	7.4 minutes (*trans*-metconazole)

The GC/MSD system was not linear over the range required for the analyses, therefore a weighted polynomial curve fit was used to calculated the residue amounts. The data from the standard analyses was entered into an Excel® spreadsheet, using a 1/concentration weighting factor and using the standard concentration (µg/mL) for the y-axis, and the response as the x-axis. A curve trend line was generated, and the equation for the curve was determined ($y = Ax^2 + Bx + C$, where A, B, and C are constants). Sample amounts (as µg/mL) were determined by substituting the sample area responses into the curve equation for the x value, and solving for y. Parts per million amounts were determined from the µg/mL sample amounts by dividing by the final sample concentration (g/mL). The correlation coefficient for the standard curve is required to be at least 0.995, and the standard amounts, when back calculated from the curve are expected to be within 15% of the nominal concentration for a set to be acceptable.