ABSTRACT

The objective of this validation study was to demonstrate the applicability and repeatability of method BASF Analytical Method No. D1410 (L0257/01) for the determination of residues of the geometric isomers of dimethomorph (BAS 550 F), *E*-dimethomorph (Reg. No. 4110868) and *Z*-dimethomorph (Reg. No. 4110869), in drinking (tap) water and surface (pond) water by LC-MS/MS.

Principle of the method. The residues of dimethomorph in water samples are analyzed by direct injection onto a high performance liquid chromatography (HPLC) column with detection by positive ion electrospray ionization tandem mass spectrometry (MS/MS-ESI), monitoring ion transitions at m/z 388→301 (proposed as the primary transition for quantitation) and m/z 388→165 (typically for confirmatory purposes) for both isomers. The isomers are separated by their retention times on the HPLC column, enabling quantitation of the contribution of each analyte. The results are calculated by direct comparison of the sample peak responses to those of external standards.

Test conditions. For validation, untreated drinking (tap) water and surface (pond) water samples were fortified with dimethomorph (both isomers) and analyzed according to the established method validation guidelines. The analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.05 µg/kg (ppb), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 0.5 µg/kg. For each analyte, the two mass transitions described above were evaluated. In conjunction with the subject study, matrix- and solvent-matched standards were analyzed in a separate experiment to evaluate any potential matrix effects.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The LOQ was defined by the lowest fortification level successfully tested. The validated LOQ for residues of dimethomorph in water is 0.05 µg/kg, for each analyte, which corresponds to a concentration in the final volume of 0.05 ng/mL. The LOD for each analyte in water was set at 20% of the LOQ, or 0.01 µg/kg, which corresponds to 0.01 ng/mL.

Selectivity. The method determines residues of the geometric isomers of dimethomorph, *E*- and *Z*-dimethomorph, in water by LC-MS/MS. No interfering peaks were found at the retention times for these analytes. The experiment to evaluate any potential matrix effects showed that the matrix load in the samples from the each water type had no significant influence on analysis (matrix effects < 20%); therefore, the validation samples were analyzed only using solvent-based calibration standard solutions.

Linearity. Acceptable linearity was observed for the standard range and the two mass transitions tested for each analyte: The method-detector response was linear over the 0.01 to 0.25 ng/mL range ($r = \geq 0.9975$).

Standard stability. The stability of dimethomorph in standard solutions has been determined. In a previous study, dimethomorph was shown to be stable in methanol, the solvent used for preparation of stock and intermediate standard solutions, for at least 3 months, under refrigeration (Reference 3). In conjunction with the subject study, both isomers of dimethomorph were shown to be stable in calibration standards prepared in water for at least 11 days when stored under refrigeration. During the course of this study, all solutions were used within the demonstrated time period of stability.

1. INTRODUCTION

1.1 Background and Purpose of Study

Dimethomorph, (E,Z) 4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)-1-oxo-2-propenyl] morpholine, is a systemic fungicide which has as its mode of action the inhibition of sterol (ergosterol) synthesis and was developed to control various diseases (downy mildews, late blights, crown and root rots) in fruits and vegetables. The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1410 (L0257/01, Reference 1) for the determination of residues of the geometric isomers of dimethomorph, *E*- and *Z*-dimethomorph, in drinking water and surface water by LC-MS/MS.

1.2 Principle of the Method

Using BASF Analytical Method No. D1410, residues of *E*- and *Z*-dimethomorph in water are separately identified and quantified as individual compounds by direct injection of an aliquot of the water sample using LC/MS/MS. No additional sample preparation or clean-up are required.

1.3 Specificity/Selectivity

The residues *E*- and *Z*-dimethomorph are determined by high performance liquid chromatography (HPLC) positive ion electrospray ionization tandem mass spectrometry (MS/MS-ESI), monitoring ion transitions at m/z 388→301 (proposed as the primary transition for quantitation) and m/z 388→165 (typically for confirmatory purposes) for both isomers. The isomers are separated by their retention times on the HPLC column, enabling quantitation of the contribution of each analyte. The results are calculated by direct comparison of the sample peak responses to those of external standards.

As HPLC/MS/MS is regarded as a highly-specific detection method when two ion transitions have been validated, an additional confirmatory method or technique is not necessary.

2. MATERIALS AND METHODS

2.1 Test systems

The drinking and surface water samples used in this study were tap water sample number 710640-01TW and pond water sample number 710640-02PW. These samples had been collected locally (at the test facility property) and were characterized by Agvise Laboratories under a previous BASF study (Reference 2). The GLP water characterization reports are provided in Appendix G.

All samples were received at ambient temperature from the field and were stored in a refrigerator (< 10 °C) at BASF Crop Protection prior to analysis. Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 724058-1). The test system samples were assigned unique numbers according to SOP 10.04.XX and these were recorded in each analytical set or "Master Sheet" (e.g., tap water fortification sample 724058-2-4, from Master Sheet No. 724058-02). The actual sample numbers used for the analysis were identified in the raw data and in this final report.

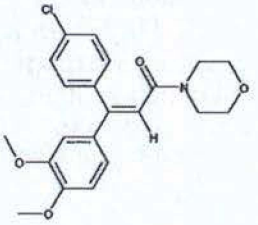
2.2 Test and Reference Items

The test/reference standards shown below were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference items were maintained frozen until use in this study. BASF Aktiengesellschaft determined characterization and purity prior to the substances being used in this study. Details of these determinations are available to BASF and are located at Landwirtschaftliche Versuchsstation der BASF, Limburgerhof, Germany.

2.2.1 E-dimethomorph

BAS-Code	BAS 550 F
Common Name	*E*-Dimethomorph
IUPAC Name	(*E*)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)-acryloyl]morpholine
BASF Reg. No.	4110868
CAS-No.	113210-97-2
Molecular Formula	$C_{21}H_{22}ClNO_4$
Molecular Weight	387.86 g/mol
Lot No.	AC11187-92
Purity (%)	98.9
Test Substance Type	Pure active ingredient (PAI)
Storage Advice	Refer to "Certificate of Analysis" Appendix A (page 24)
GLP	Yes
Expiration Date	July 1, 2021

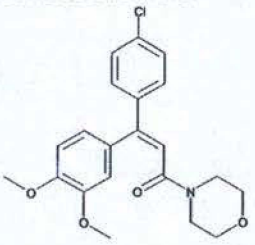
Chemical Structure:



2.2.2 Z-dimethomorph

BAS-Code	BAS 550 F
Common Name	*Z*-Dimethomorph
IUPAC Name	(*Z*)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)-acryloyl]morpholine
BASF Reg. No.	4110869
CAS-No.	113210-98-3
Molecular Formula	$C_{21}H_{22}ClNO_4$
Molecular Weight	387.86 g/mol
Lot No.	AC7467-013
Purity (%)	97.3
Test Substance Type	Pure active ingredient (PAI)
Storage Advice	Refer to "Certificate of Analysis" Appendix A (page 24)
GLP	Yes
Expiration Date	July 1, 2019

Chemical Structure:



The test/reference items were used in the study to generate data for both instrument and method performance. Quantitation of residues in all samples was achieved using calibration curves calculated by linear regression of instrument responses for the reference items. The performance of the instrument was evaluated during each injection set.

2.3 Route of Administration

In this method validation study, the test items were applied to the test system as mixed analytical standard solutions (in methanol) by micropipette to ensure precise delivery of a small amount of the test items.

2.4 Validation of Method

For validation, untreated drinking (tap) water and surface (pond) water samples were fortified with dimethomorph (both isomers) and analyzed according to the established method validation guidelines. To test the repeatability of the method, the analytical sets for each matrix typically consisted of a reagent blank, two controls, five replicates fortified with analyte at the method limit of quantitation, 0.05 µg/kg (ppb), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 0.5 µg/kg. For each analyte, the two mass transitions described above were evaluated.

2.5 Analytical Materials and Procedures

The method used in this method validation study was BASF Analytical Method No. D1410. The analytical method, as performed in this method validation study, is described in detail in this section.

2.5.1 Equipment

Equipment	Size, Description	Manufacturer
Balance, Top Loader	Model PJ3600	Mettler DeltaRange
Balance, Analytical	Model AT100	Mettler
Beakers	Various	PYREX Brand, VWR
Volumetric pipettes	0.5 mL, 1 mL	Fisher Scientific – Class A
MicroMan pipettes	10-1000 µL	Gilson
Pasteur Pipet, disposable	Various	VWR
Volumetric Flasks, Various	100, 50, 25 ,10 and 5 mL	Various
LC Vials	2 mL injection vials	Waters
LC	Acquity UPLC	Waters
MS/MS	API 5500	AB Sciex
HPLC Column	Atlantis T3 (100 x 2.1mm, 3µ)	Waters
Vortex Mixer	Genie 2	Fisher Scientific

2.5.2 Reagents and Chemicals

Chemical	Grade	Manufacturer/Supplier
Methanol	HPLC Grade	EMD
Formic acid	98% GR ACS	EMD
Water, e.g. Baker® or Millipore®	HPLC Grade	BDH ARISTAR PLUS

2.5.2.1 Solutions and Solvent Mixtures

The following solutions and solvent mixtures are used in the method.

Description	Composition
HPLC mobile phase A	0.1% Formic Acid in Water Add 1000 mL of water and 1 mL of concentrated formic acid into a 1 L Erlenmeyer and mix well to ensure a completely homogeneous solution.
HPLC mobile phase B	0.1% Formic Acid in Methanol Add 1000 mL of methanol and 1 mL of concentrated formic acid into a 1 L Erlenmeyer and mix well to ensure a completely homogeneous solution.

The solutions are prepared in different volumes, if needed, provided that the proportions are not modified.

2.5.2.2 Standard Solutions

Stock Solutions

To prepare the stock solutions, *E*- and *Z*-dimethomorph analytical standards (5 mg each) are weighed into separate volumetric flasks, dissolved, and then and brought to volume (5 mL) with methanol. To ensure a completely homogeneous solution, the solutions are sonicated or vortexed. The stock solutions (1.0 mg/mL) are transferred into amber glass bottles and stored under refrigeration.

As the purity of the standards used in this study were >95% (*E*-dimethomorph, 98.9%; and *Z*-dimethomorph, 97.3%), correction for purity of the neat standards was not performed.

Fortification Solutions

The fortification solutions are prepared by diluting the stock solutions, described above, volumetrically with methanol according to the following dilution scheme:

Take solution (µg/mL)	Volume (mL)	Final volume (mL)	Concentration (µg/mL)
1000	0.5*	50	10
10	0.5	50	0.1
0.1	2.5	50	0.005

*0.5 mL of each stock solution
A complete, homogeneous solution is ensured by sonication or vortexing (as needed).

Calibration Standard Solutions

The calibration standard solutions used for LC/MS/MS analysis are prepared by diluting the fortification solutions, described above, volumetrically with water, according to the following dilution scheme:

Take solution (ng/mL)	Aliquot Volume (mL)	Dilute with water to final volume (mL)	Concentration (ng/mL)
5	2.5	50	0.25
5	1	50	0.1
5	0.5	50	0.05
0.25	5	50	0.025
0.1	5	50	0.01

A complete, homogeneous solution is ensured by vortexing (as needed).

Matrix Matched Calibration Standard Solutions

During method development, it was demonstrated that the matrix load in the water samples had no significant influence on the analysis; therefore, samples could be analyzed using calibration standard solutions prepared in solvent alone. Matrix-matched calibration standards are used for quantitation when signal suppression or enhancement, typically >20% compared to the response for standards prepared in calibration solution alone, is observed due to the matrix load in the sample. In the event that matrix-matched standards, also referred to as "instrument recovery samples", are needed for successful analysis, calibration standard solutions are prepared in matrix solution (in this case, control tap or surface water). Matrix-matched standards are prepared in a way that the matrix load is at least 90% of the matrix load in the unknown samples. In addition, the matrix load are to be the same in all calibration standard solutions.

Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data for this study.

2.5.3 Stability of Test and Reference Items in Solution

2.5.3.1 Stability of Stock and Fortification Standard Solutions

As noted in the preceeding sections, stock solutions of E- and Z-dimethomorph are prepared in methanol (1 mg/mL) and are typically made fresh every 3 months. The mixed intermediate and fortification solutions containing each isomer of dimethomorph are prepared by combining aliquots of the stock solutions for each analyte and diluting with methanol. The fortification standards are typically made fresh every 1 month. Dimethomorph has been shown to be stable in stock solutions held under refrigeration for 3 months in a previous study (Reference 3). The test/reference substance solutions were stored in a refrigerator during their use in this study.

2.5.3.2 Stability of Calibration Standard Solutions

The calibration standards are prepared by serial dilution of the intermediate standatd solutions (described above) using water and are typically made fresh every 1 month. To determined the stability of each analyte in the calibration standards, the standards used to prepare the calibration curve for the method validation set in this study were analyzed against freshly prepared standard solutions.

2.5.3.3 Stability of Extracts

There are no "extracts" in this method, which consists of the direct injection of the water samples onto the HPLC column; however, as noted in the previous sections and based on the results obtained in this study, each analyte is stable in water at various concentrations when held under refrigeration.

2.5.4 Analytical Procedure

No preparation of water samples is necessary prior to analysis. "Unknown" samples are stored unfiltered, and refrigerated in the dark until analysis.

2.5.4.1 Weighing and Fortification

An aliquot (1 mL) of the sample is transferred into a screw-top injection vial with PTFE-lined caps for analysis by means of an accurate pipette. Cloudy/turbid samples or samples with

visible particulate matter are filtered (if needed) using a syringe filter prior to or during the transfer to injection vials.

For fortification experiments, mixed fortification standards containing both geometric isomers of dimethomorph are added to the matrix according to the scheme shown below. After fortification, the fortified samples are thoroughly homogenized by vortexing.

Sample Type	Sample Vol. (mL)	Spiking Solution Conc. (ng/mL)	Vol. of Spiking Solution (mL)[1]	Level of Fortification (ppb)
Control	1	-	-	0
Fortification (LOQ)	1	5	0.01	0.05
Fortification (10xLOQ)	1	50	0.01	0.5
Treated	1	-	-	-

1. Volume of spiking solution added to generate the fortified sample should not exceed 10% of sample weight or volume (plant and animal) and 1% in case of water and soil..
2. Different concentration schemes can be used, if different fortification levels are required. Total volume of solutions prepared can be changed, if overall ratios are maintained.

2.5.4.2 Sample Extraction

No extraction is required. The samples are injected directly on the HPLC column.

2.5.4.3 Sample Clean-Up and Preparation for Analysis

No clean-up steps are required. Once vialed and capped as described above the samples are ready for analysis. For higher fortification levels or in the case of significant matrix effects, an appropriate dilution in water may be necessary.

2.5.5 Instrumental Analysis

2.5.5.1 Calibration Procedures

A sequence for measurement generally consists of calibration standards; control samples; procedural recovery samples; unknown samples; and/or instrument recovery samples. At least five calibration standard concentrations are typically included in an analytical set. Reagent blanks or blanks are also typically included, as needed. Each injection set begins and ends with the injection of a calibration standard; standards are to be interspersed with samples; and each calibration standard should be at least injected twice.

In this study, calculation of results was based on peak area measurements using a calibration curve consisting of five calibration standard levels for each analyte. The calibration curve was obtained by direct injection of standards for LC-MS/MS, prepared with *E*- or *Z*-dimethomorph, in the concentration range of 0.01 to 0.25 ng/mL. In all injection runs, the same injection volume was used for all samples and standards.

2.5.5.2 Instrumentation and Conditions

	Parameter		
Chromatographic System	Acquity UPLC with Autosampler		
Guard-column	N/A		
Analytical-column	Atlantis T3 (100 mm x 2.1 mm, 3µm)		
Column Temperature	45°C		
Injection Volume	50 µL*		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Divert Valve	N/A		
Time to Waste	N/A		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.0	66	34
	0.25	66	34
	2.0	30	70
	5.75	1	99
	6.4	1	99
	6.5	66	34
	7.0	66	34
Detection System	AB Sciex API 5500 Mass Spectrometer		
Ionization	Electrospray (ESI)		
Ionization Temperature	550 °C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Dimethomorph (BAS 550 F) *E*-isomer (4110868)	388 --> 301** 388 --> 165	positive	approx. 3.15 min
Dimethomorph (BAS 550 F) *Z*-isomer (4110869)	388 --> 301** 388 --> 165	positive	approx. 3.27 min

*Injection volume can be modified to suit instrument's sensitivity
** proposed as quantification transition. Any of these transitions could be used for quantitation.

2.5.5.3 Calculation of Residues and Recoveries

The calculation of residue results is based on peak area measurements. Example residues calculations are provided in Appendix B. In this study, for the procedural recoveries, the sample weight was considered to be exactly "1.000 g" for the calculation of residues. In the laboratory, the samples were measured to great precision for the fortification samples. The recovery is the percentage of the fortified amount which is recovered through the method and the weights cancel out during the final calculation step; therefore, the use of the rounded figure (1.00 g) in calculations has no impact on the recovery values reported.

2.5.5.4 Influence of Matrix effects on Analysis

In conjunction with the subject study, matrix-matched standards and solvent-based standards were analyzed in a separate experiment to evaluate any potential matrix effects on LC/MS/MS analysis. This involved comparing calibration standards prepared in blank matrix against calibration standard solutions prepared in HPLC grade water. The matrix-matched standards were made by diluting 10 µL each of three mixed standards (concentrations, 2.5, 5, and 10 ng/mL each analyte) with control tap or surface water to a 1 mL final volume. The final concentrations achieved for these matrix-matched standards were 0.025, 0.05, and 0.1 ng/mL, representing ½ LOQ, LOQ, and approximately 2X LOQ. Each set of matrix-matched standards

(for each water type) was bracketed by a block of calibration standards and was to have an additional single injection of each of the tested standard levels (0.025, 0.05, and 0.1 ng/mL) occur during the run. Only the standards which immediately bracket a matrix set, and all standard injections within that matrix set, were used in calculations involving matrix effects.

The data generated were evaluated by comparing the average area response of the standards for at least three injections of each type (with and without matrix) for each of the three standard concentration levels. Acceptability (i.e., matrices had no significant influence on the analysis) requires a difference in area of <20% and is calculated for each standard concentration for each matrix using the following equation:

Mean Area Count (instrument response) Change (%) =

$$\left[\left(\frac{Mean\ area\ (solvent\text{-}based\ standards) - Mean\ area\ (matrix\text{-}matched\ standards)}{Mean\ area\ (solvent\text{-}based\ standards)}\right)\right] x100$$

For each analyte/ matrix/ion transition, an overall average "Mean Area Change (%)" across the three tested concentrations is also calculated to make a general assessment of acceptability with respect to matrix effects.

Figure 1. Method Flowchart – Analysis of Dimethomorph in Water

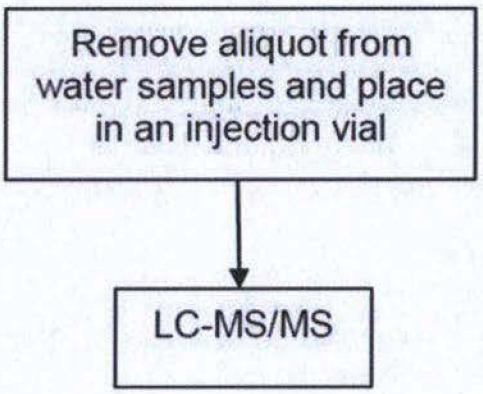


Figure 2. Typical Recovery Calculation for LC/MS/MS Quantitation

Sample No. 724058-2-4. Control drinking (tap) water fortified at the LOQ with dimethomorph (both isomers), Master Sheet No. 724058-2.

$$\text{Concentration of analyte (ng/mL)} = \frac{\text{peak area - intercept}}{\text{slope}}$$

	E-Dimethomorph	*Z*-Dimethomorph
Peak Area =	81,909.0	118,605.0
Intercept =	4190.9062	6073.3362
Slope =	1697455.8942	2393870.4340
Conc. (ng/mL) =	0.0458	0.0470

The concentration of dimethomorph in µg/kg is calculated as shown in equation:

$$\text{Residue [µg/kg]} = \frac{V_{end} \times C_A}{G \times A_F}$$

Where:

V_{end} = Final volume [mL]
C_A = Concentration of analyte as read from the calibration curve [ng/mL]
G = Weight of the sample extracted [1.00 grams]
A_F = Aliquotation factor

	E-Dimethomorph	*Z*-Dimethomorph
V_{end} =	1 mL	1 mL
A_F =	100%	100%
Conc. (ng/mL) =	0.0458	0.0470
Residue (ppb) =	0.0458	0.0470

Net residue (ppb of analyte) = Residue (ppb of analyte) - Residue in Control (ppb)

$$\text{Recovery of analyte (\%)} = \frac{\text{Residue (ppb of analyte) - Residue in Control (ppb)}}{\text{Amount Fortified (ppb)}} \times 100$$

	E-Dimethomorph	*Z*-Dimethomorph
Amount fortified	0.05	0.05
Residue (ppb) =	0.0458	0.0470
Residue in control =	0.0000	0.0000
%Recovery	91.6%	94.0%

Use full computer/calculator precision in any intermediate calculations. Round only the final value.