1. INTRODUCTION

1.1 Background and Purpose of Study

Metconazole (BAS 555 F) is a fungicide active against various diseases found in agricultural crops and was developed by BASF for agricultural use. The objective of this validation study was to demonstrate the applicability and repeatability of BASF Analytical Method No. D1501 for the determination of residues of metconazole in drinking water and surface water by LC-MS/MS.

1.2 Principle of the Method

Using BASF Analytical Method No. D1501/01, residues of metconazole in water samples (10 mL each) are directly injected, and the residues of metconazole are determined by HPLC-MS/MS, described below.

1.3 Specificity/Selectivity

The residues of metconazole are determined by high performance liquid chromatography (HPLC) column with detection by electrospray ionization tandem mass spectrometry (MS/MS-ESI), monitoring ion transitions (proposed as the primary transitions for quantitation) at m/z $320 \rightarrow 70$ for metconazole and, typically for confirmatory purposes, monitoring ion transitions at m/z $322 \rightarrow 70$ for metconazole. The results are calculated by direct comparison of the sample peak areas 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 well water sample number " 20141105-drinking-Well" and lake water sample number " 20141104-surface-JL". These samples had been collected locally and were characterized by Agvise Laboratories. The GLP water characterization reports are provided in Appendix H.

All samples were received at ambient temperature and were stored at room temperature 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., 427928-2). 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., surface water fortification sample 427928-2-4, from Master Sheet No. 427928-2). The actual sample numbers used for the analysis were identified in the raw data and in this final report.
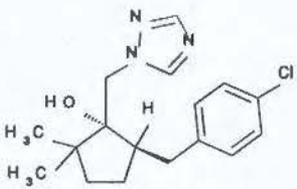
2.2 Test and Reference Items

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. If no optical purity is stated on COA, reference material is a racemic mixture of possible enantiomers.
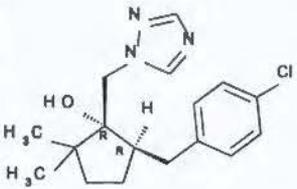
2.2.1 Cis-Metconazole
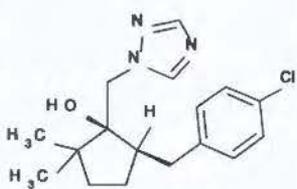
BAS-Code	555 F
Common Name	Cis-Metconazole
IUPAC Name	Cis-1-(1H-1,2,4-triazole-1-ylmethyl)-2,2-dimethyl-5-(4-chlorobenzyl)cyclopentanol
BASF Reg. No.	4079468
CAS-No.	115850-27-6
Molecular Formula	$C_{17}H_{22}ClN_3O$
Molecular Weight	319.8
Lot Number	AC8879-136A
Purity	99.3%

2.2.2 Trans-Metconazole

BAS-Code	555 F
Common Name	Trans-Metconazole
IUPAC Name	Trans-1-(1H-1,2,4-triazole-1-ylmethyl)-2,2-dimethyl-5-(4-chlorobenzyl)cyclopentanol
BASF Reg. No.	4079654
CAS-No.	115850-28-7
Molecular Formula	$C_{17}H_{22}ClN_3O$
Molecular Weight	319.8
Lot Number	AC9339-122A
Purity	99.1%

2.2.3 M555F000-RR

Metabolite-Code	M555F000-RR
IUPAC Name	(1R,5R)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
BASF Reg. No.	5836048
CAS-No.	115850-28-7
Molecular Formula	$C_{17}H_{22}ClN_3O$
Molecular Weight	319.8
Lot Number	L84-124
Purity	97.4%
Optical Purity	99.71%

2.2.4   M555F000-SR

Metabolite-Code	M555F000-SR
IUPAC Name	(1S,5R)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
BASF Reg. No.	4677200
CAS-No.	115850-27-6
Molecular Formula	$C_{17}H_{22}ClN_3O$
Molecular Weight	319.8
Lot Number	L84-108
Purity	99.5%
Optical Purity	100.0%

2.2.5   M555F000-RS

Metabolite-Code	M555F000-RS
IUPAC Name	(1R,5S)-5-(4-chlorobenzyl)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
BASF Reg. No.	5836046
CAS-No.	127307-20-4
Molecular Formula	$C_{17}H_{22}ClN_3O$
Molecular Weight	319.8
Lot Number	L84-110
Purity	99.1%
Optical Purity	99.59%

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.

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

For validation, untreated drinking (well) water and surface (lake) water samples were fortified with each analyte 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, 25 ng/L (ppt), and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 250 ng/L. For each analyte, the two mass transitions described above were evaluated.

The method used in this validation study was BASF Analytical Method No. D1501 and it was performed as written. The text and tables of the method are reproduced in this section with only minor changes (for readability or clarity) to allow this document to serve as a working method for laboratories to perform residue analysis. Any relevant substitutions or modifications (if any) were documented in the raw data and are described in this final report.

2.3 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Beakers	Various Sizes	PYREX Brand, VWR Scientific Products	13922-029
Culture tube caps	16 mm	VWR	60828-768
Culture Tubes	Glass, disposable, 16x100mm size	VWR	47729-576
Cylinder, Graduated	Various sizes	Various	----
HPLC Column	Chiralpak AD-3R, 2.1 x 150 mm, 3 μm	Diacel	19894
LC	Acquity UPLC	Waters	--
LC Vials	2 mL injection vials	National Scientific	C400-79
MicroMan pipettes	10-1000 μL	Gilson	M-25, M-50, M-250, M-1000
MS/MS	API 5500	AB Sciex	----
Ultrasonic Bath	Model FS 7652H	Fisher Scientific	----
Various Flask, Volumetric	100, 50, 25 ,10 and 5 mL	Various	----
Volumetric pipettes	Various sizes	VWR	-
Vortex mixer	Genie 2	VWR	58816-121

2.4 Reagents

2.4.1 Chemicals

Chemical	Grade	Manufacturer/Supplier
Methanol	Gradient Grade	Merck, Darmstadt, FRG
Formic acid	>95%	Sigma-Aldrich
Water, e.g. Baker® or Millipore®	HPLC Grade	BDH ARISTAR PLUS

2.4.2   Solutions and Solvent Mixtures

Description	Code	Composition
HPLC mobile phase A	LC1	0.1% Formic Acid in Water Add 1000 mL of water and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.
HPLC mobile phase B	LC2	0.1% Formic Acid in Methanol Add 1000 mL of Methanol and 1 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

2.4.3   Standard Solutions
2.4.3.1 BAS 555 F Isomers

Stock Solution

Standard Containing Single Isomer (e.g., (S,S)-metconazole)

Prepare a stock solution containing 1 mg/mL by weighing an appropriate amount of reference item into a volumetric flask and add the required volume.

For example, weigh 10 mg of a single isomer of metconazole (e.g. (S,S)-metconazole) into a 10-mL volumetric flask. Dissolve and dilute to mark with methanol. This creates a solution containing 1 mg/mL of (S,S)-metconazole. Ensure a complete homogeneous solution (e.g., by sonication and/or vortexing).

Standard Containing More Than One Diastereomeric Form (e.g., (+/-)-cis-metconazole)

Prepare a stock solution containing 1 mg/mL of each isomer individually by weighing an appropriate amount of each reference item into a single volumetric flask and add the required volume.

For example, weigh 20 mg (+/-)-cis-metconazole into a 10-mL volumetric flask. Dissolve and dilute to mark with methanol. This creates a solution approximately 1 mg/mL of each diastereomeric form (to calculate absolute concentration see substance Certificate of Analysis for isomeric ratio). Ensure a complete homogeneous solution (e.g., by sonication and/or vortexing). The stock solutions for other mixed standards are made in a similar fashion.

Standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved for example using one of the following approaches:
1. Two stock solutions are independently prepared. One is used for preparation of fortification solutions, the other for calibration standard solutions.
2. Fortification and calibration standard solutions should be prepared from one stock solution in separate dilution series.
For subsequent preparations of solutions, freshly prepared solutions can be compared directly to previous standard solutions.

A correction for purity is done if the purity is ≤ 95%. If the purity is > 95 % correction is optional.

Fortification Solutions

Prepare mixed standard solutions for fortification by combining stock solutions of each analyte (see above) in a flask, e.g. screw cap Supelco vials allowing storage without solvent loss. Dilute, e.g. volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of mixed Fortification solutions

Take solution (µg/mL)	Aliquot Volume (mL)	Dilute with methanol to a final volume of (mL)	Final Concentration (µg/mL)
1000	0.5 (of each solution)	50	10
10	5	50	1.0
1.0	5	50	0.1
0.1	5	50	0.01

Note: 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.

Additional Information :

Use amber bottles with Teflon-lined screw caps as storage containers for all standard solutions

2.4.3.2   Calibration Standard Solutions

Prepare mixed standard calibration solutions for LC-MS/MS analysis by using the solutions that were prepared in Section "stock solutions" or "fortification solutions" in flasks. Dilute e.g. volumetrically with appropriate solvents as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of standard solutions for calibration

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with water to a final volume of (mL)	Final Concentration (ng/mL)
10	5	50	1.0
1.0	10	100	0.1
0.1	25	50	0.05
0.1	25	100	0.025
0.1	5	50	0.01
0.1	2.5	50	0.005

Note: 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.

Additional Information :

Use amber bottles with Teflon-lined screw caps as storage containers for all standard solutions

2.4.4 Stability of Test and Reference Items in Solution

2.4.4.1 Stability of Stock Solutions

As noted in the preceding sections, stock solutions (1 mg/mL) are prepared in methanol. The stability of metconazole in stock solutions has been determined. In previous studies, metconazole was shown to be stable in stock solutions prepared in methanol for at least 3 months, when held under refrigeration (Reference 1).

2.4.4.2 Stability of Fortification and Calibration Standard Solutions

The mixed fortification and calibration standard solutions are prepared in methanol or water, as described above, and are typically made fresh every 1 month. The stability of each analyte in fortification solutions has been determined. In previous studies, metconazole, was shown to be stable in fortification solutions prepared in methanol for at least 1 month, when held under refrigeration (Reference 1). In this study, metconazole was proven stable in standards prepared in water and refrigerated for at least 1 month.

During the course of this study, the test/reference substance solutions were stored in a refrigerator and all solutions were used within the demonstrated time period of stability:

Analyte	Standard	Solvent	Duration (days)
M555F000-RR	Stock	Methanol	33[1]
	Fortification	Methanol	33[1]
	Calibration	Water	31[2]
M555F000-RS	Stock	Methanol	33[1]
	Fortification	Methanol	33[1]
	Calibration	Water	31[2]
M555F000-SR	Stock	Methanol	33[1]
	Fortification	Methanol	33[1]
	Calibration	Water	31[2]
M555F000-SS	Stock	Methanol	33[1]
	Fortification	Methanol	33[1]
	Calibration	Water	31[2]

[1] BASF Study 423586 (Reference 1)
[2] Determined in conjunction with the subject study, BASF Study 427928

2.4.4.3 Stability of Extracts

The method validation fortification sample extracts were stored under refrigeration (if needed) prior to analysis and were analyzed within 1 day of preparation. There is no stability of extracts (as the water samples are not extracted per se). The stability of analytes in final volume, determined in conjunction with the subject study, is shown below:

Analyte	Water Extracts	Duration (days)
	Drinking Water	7
Metconazole	Surface Water	7

3. ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples have to be sufficiently mixed beforehand, in order to assure that the aliquot taken for residue analysis is representative for the whole sample.

3.2 Sample Storage

Liquids samples are not stored filtered in order to include analytes adsorbed to particles and to avoid potential filtration losses due to filter sorption. Until analysis, water samples are stored in clean amber glass bottles in a refrigerator or frozen conditions.

3.3 Weighing and Fortification

For treated samples and control samples, measure 10±0.1 g (or 10 mL) of water sample into a culture tube.

For fortified samples, measure 10±0.1 g (or 10 mL) of water sample into a culture tube. Fortify the solution with analyte(s) and mix well.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification per Isomer
Control	10 g (or mL)	-	-	0.00 ng/L
Fortification (LOQ)	10 g (or mL)	10 ng/mL	0.025 mL	25 ng/L *
Fortification (10xLOQ)	10 g (or mL)	100 ng/mL	0.025 mL	250 ng/L
Treated	10 g (or mL)	-	-	--

* limit of quantification

> Note: 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.

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.

3.4 Preparation for Measurement

Transfer an aliquot of sample to LC vial for analysis. High residue samples may be diluted with DI water.

3.5 Influence of matrix effects on analysis

The method for the analysis of metconazole is a direct injection method with no sample extraction or clean-up. Based on this method, the procedural recovery samples also serve as instrument recovery samples for the purpose of evaluating the influence of matrix effects on LC-MS/MS analysis. Since the mean recoveries of the low fortification samples in surface and drinking water matrices were within acceptable ranges (70-110%), this also serves to demonstrate the matrix load in the samples had no influence on the analysis. Furthermore, there is little difference (<20 %) between the average recoveries of the high fortification and the low fortification samples, which serves to support this conclusion.

4. QUANTIFICATION AND CALCULATION

4.1 Set-up of the analytical run

A sequence for measurement generally consists of:
o Calibration standards
o Control samples
o Procedural recovery samples
o Unknown samples
o Instrument recovery sample

Reagent Blanks or blanks can also be injected if necessary. Each injection set should begin and end with an injection of a calibration standard. Standards should be interspersed with samples. Each calibration standard should be at least injected twice. At least 5 calibration levels need to be injected.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions

	Parameter		
Chromatographic System	Waters UPLC[1]		
Analytical-column	Chiralpak AD-3R, 2.1 x 150 mm, 3 µm		
Column Temperature	40°C		
Injection Volume	100 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, Methanol / formic acid,	1000/1, v/v 1000/1, v/v	
Flow Rate	150 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0	15	85
	14	15	85
Detection System	AB Sciex 5500 Mass Spectrometer		
Ionisation	Electrospray (ESI)		
Analyte	Transitions	Polarity	Expected Retention Time
M555F000-RS Reg. No. 5836046	320 –> 70* 322 –> 70	positive	Approx. 7.8 min
M555F000-SR Reg. No. 4677200	320 –> 70* 322 –> 70	positive	Approx. 8.6 min
M555F000-RR Reg. No. 5836048	320 –> 70* 322 –> 70	Positive	Approx. 10.6 min
M555F000-SS Reg. No. 5836048	320 –> 70* 322 –> 70	positive	Approx. 11.5 min

* proposed as quantification transition. Any of these transitions could be used for quantitation

[1] The system is a UPLC instrument. However, the method operates under HPLC conditions (<400 bar).

> Note: Instruments with similar specifications may substitute the equipment listed above. The instruments used are applicable for analysis if the recoveries of the fortification experiments are in the acceptable range.
>
> A divert valve can be used to reduce the matrix load on the detection system.

Instrument conditions, e.g. injection volume, column, gradient steps may be modified; however changes have to be documented in the raw data. Changes are acceptable, if the recoveries of the fortification experiments are in the acceptable range of the required guidelines.

If the use of different analytical columns (different stationary phase)is required, then methodology has to be validated by analyzing at least five replicates of fortified samples prepared at e.g. LOQ and 10xLOQ. Assessment of matrix impact by preparation of at least one concentration level of a matrix matched standard is also required.

The same applies to different mass transitions used: Validation of the methodology is required as described above (fortification and assessment of matrix effect).

Other parameters, such as ion source gas flows and voltages, are highly specific of the equipment used and therefore not listed. Those parameters may need to be adapted to the actual instrument.

4.2.2 Calibration procedures

Calculation of results is based on peak area measurements using a calibration curve. At least 5 calibration levels need to be injected (e.g., required for enforcement). The calibration curve is obtained by direct injection of BAS 555 F standards for LC-MS/MS in the range of 0.1 ng/mL to 0.005 ng/mL. In a given injection run, the same injection volume is used for all samples and standards.

Linear calibration functions are preferred for evaluation. If other functions are used (e.g. quadratic), this should be fully justified.

4.2.3 Calculation of Residues and Recoveries

Calculation of results is based on area measurements.
For the procedural recoveries, a sample volume of 10 g (or 10 mL) will be considered in the final calculation of residues [ng/L]. This approach requires that the sample volume has to be within a measuring precision of 10±0.1 mL for fortification samples (matrix). The recovery is the percentage of the fortified amount of the analyte (µg or ng), which is recovered after the entire sample work-up steps.

Calculation is described by the equation given below:

The residues of BAS 555 F in ng/L are calculated as shown in equations I and II:

I. Concentration [ng/mL] $$= \frac{Response - Intercept}{Slope} = C_A$$

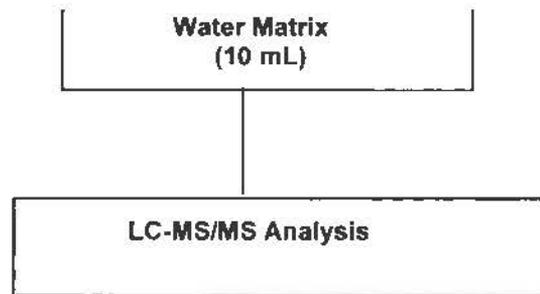II. Residue [ng/L] $$= \frac{V_{end} \times C_A}{G}$$

V_{end} = Final volume of the extract after all dilution steps [mL]
C_A = Concentration of analyte as read from the calibration curve [ng/mL]
G = Volume of sample extracted in L

The recoveries of spiked compounds are calculated according to equation III:

III.   **Recovery %** $= \dfrac{(\text{Residue in fortified sample} - \text{Residue in control}) \times 100}{\text{Amount of analyte fortified}}$

Example residues calculations are provided in Appendix B.

Figure 1. Analysis of BAS 555 F in water

8. SUMMARY OF METHOD

Type of method:	Method Validation by LC-MS/MS
Test systems:	Drinking water (well water) and surface water (pond water)

Analyte mass transitions (m/z):

M555F000-RR Quantitation transition: m/z 320 → 70
M555F000-RR Confirmatory transition: m/z 322 → 70
M555F000-RS Quantitation transition: m/z 320 → 70
M555F000-RS Confirmatory transition: m/z 322 → 70
M555F000-SR Quantitation transition: m/z 320 → 70
M555F000-SR Confirmatory transition: m/z 322 → 70
M555F000-SS Quantitation transition: m/z 320 → 70
M555F000-SS Confirmatory transition: m/z 322 → 70

Selectivity: The tested matrices showed no significant interference peaks (< 20%) at the retention time of the analytes. The experiment to evaluate any potential matrix effects, as well as the method validation analyses, showed that the matrix load in the samples had no influence on analysis.

Justification of selection of ions: Instrument parameters obtained by direct infusion of a solution of the analyte and the MRM product ions are selected from Q1 scan of each compound. The Q1 scan and the product ion spectra are shown in Appendix GAppendix G.

Analytical procedure: The residues of the enantiomers of metconazole are analyzed in water (10 mL) by direct injection of the sample and quantification using LC-MS/MS in positive ion mode.

Confirmatory technique: Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary. Quantification is based on the monitoring of two mass transitions for metconazole.

Limit of detection: The limit of detection was estimated at 20% of the limit of quantification, equivalent to 5 ng/L for all analytes. The lowest standard for each analyte in the calibration curve has good detectability (signal to noise ratio greater than 3:1).

Limit of quantification (LOQ): The limit of quantification is defined as the lowest fortification level successfully tested. The limit of quantification is 25 ng/L for all analytes.

Levels of fortification: Five replicates fortified with analyte at the method limit of quantitation, 25 ng/L (ppt), and five replicates fortified at a higher level, corresponding to 10X the LOQ, 250 ng/L.

Potential Problems The glassware used for the method should be thoroughly rinsed with methanol to prevent contamination.

Safety:

The test and reference items, as well as the chemicals required for this analysis, should be handled in accordance with good industrial hygiene and safety practice. Avoid contact with the skin, eyes and clothing. Wearing of closed work clothing is recommended. Remove contaminated clothing. Ensure that work clothing is stored separately. Keep away from food, drink and animal feed stuffs. No eating, drinking, smoking or tobacco use at the place of work. Hands and/or face should be washed before breaks and at the end of the shift. Details are given in the Materials Safety Data Sheets (MSDS) of the individual substances. All procedures involving organic solvents should be performed in a well-ventilated hood. Disposal of samples and chemicals must be done in compliance with on-site safety policies and procedures.

Time required:

The analysis of one series of samples (= 13 unknown samples, 2 fortified samples for recovery experiments, 1 blank sample) requires 0.25 working days (2 hours) per laboratory assistant. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

9. COMMENTS FROM INDEPENDENT LABORATORY VALIDATION

The method was the subject of a successful independent laboratory validation (Reference 2).

The independent laboratory noted that for the analysis of metconazole, a new analytical column may require extensive conditioning in order to sufficiently separate the trans-metconazole isomeric peaks. This may require between 2 to 6 hours of conditioning with standard injections