FINAL REPORT

Study Title

Validation of Method D1612/01:
Method for the determination of BAS 595 F (Reg.No. 4378513) and Its Z-isomer (Reg.No. 5079359) in Surface and Drinking Water by LC-MS/MS

Guidelines Covered

U.S. EPA Ecological Effects Test Guideline: OCSPP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation (ILV);
SANCO/3029/99 rev 4 (11/07/2000)
SANCO/825/00 Rev 8.1 (16/11/2010)

1. INTRODUCTION

1.1 Background and Purpose of Study

The purpose of this study was to validate BASF Analytical Method No. D1612/01 for the analysis of the triticonazole(E-isomer) and its Z-isomer in surface and drinking water using LC-MS/MS

2. MATERIALS AND METHODS

2.1 Test Systems

The water samples used in this study were drinking (well) water and surface (lake) water samples, which were characterized by AGVISE Laboratories.

The samples were held under refrigeration during the experimental period. Each analysis set was uniquely identified with a Master Sheet Number, which consisted of the study number plus a unique number (e.g., 819169-01). The test system samples were assigned unique numbers and these were recorded in each analytical set or "Master Sheet" (e.g., water fortification sample 819169-1-4, from Master Sheet No. 819169-01).

2.2 Test and Reference Substances

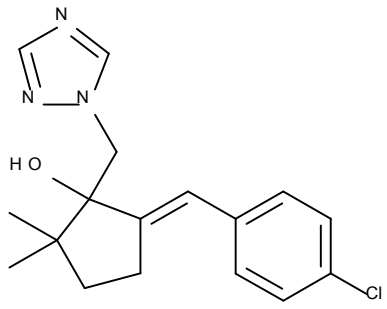
The test/reference standards shown below were synthesized by BASF Aktiengesellschaft (Limburgerhof, Germany) and used during the analytical portion of this study. The test/reference substances were maintained frozen until use in this study. BASF Aktiengesellschaft determined characterization and purity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

The test/reference substances in solution 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 substances. The performance of the instrument was evaluated during each injection set.

2.2.1 Triticonazole

Common Name	Triticonazole
BAS-Code	BAS 595 F
BASF Reg. No.	4378513
CAS-No.	138182-18-0
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8 g/mol
IUPAC Name	(RS)-(5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
Lot No.	L85-136
Purity (%)	99.4
Expiration Date	June 01, 2017

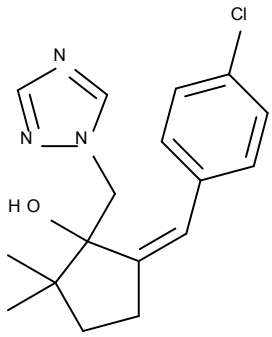
Chemical structure:



2.2.2 Triticonazole, Z-isomer

Common Name	Triticonazole
BAS-Code	M595F014 (Z-isomer)
BASF Reg. No.	5079359
CAS-No.	None assigned
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8 g/mol
IUPAC Name	(1RS)-(5Z)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol
Lot No.	BESS0578
Purity (%)	99.9%
Expiration Date	April 01, 2024

Chemical structure:



Stock solutions of triticonazole (E-isomer) and its Z—isomer were prepared in methanol. The mixed intermediate/fortification solutions containing each analyte were prepared by combining aliquots of the stock solutions for each analyte and diluting with methanol. The “precursor” calibration standards later used for preparing the matrix-matched standards used in the definitive method validation sets were prepared by serial dilution of the intermediate standards using water. The stability of the analytes in standard solutions has been determined in conjunction with this study. In this study, to determine the stability of each analyte in solution, aged standard solutions prepared in methanol or water were analyzed against freshly prepared standard solutions.

During the course of this study, the test/reference substance solutions were stored under refrigeration. Preparation and dilution data forms pertaining to the stock and working solutions are located in the raw data.

2.3 Route of Administration

In this method validation study, the test substances 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 substances.

2.4 Analytical Method

2.4.1 Principle of the Method

Using BASF Analytical Method No. D1612/01, residues of triticonazole in water are quantified using LC-MS/MS.

Briefly, residues of triticonazole in water samples (10 mL each) are filtered (0.45 µm PTFE), and then analyzed by LC-MS/MS.

2.4.2 Specificity/Selectivity

The residues of triticonazole are determined by LC-MS/MS monitoring ion transitions at m/z 318→70 (proposed as the primary transition for quantitation) and m/z 320→70 (typically for confirmatory purposes) for both isomers. The isomers are separated by their retention times on the LC 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 LC-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. The multiple reaction monitoring (MRM) transitions used to identify triticonazole were determined from the product ion scan (see Appendix J).

2.5 Validation of Method

For validation, untreated surface (lake) and drinking (well) 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 consisted of a reagent blank, and for each matrix, two controls, five replicates fortified with each analyte at the method limit of quantitation, 30 ppt, and five replicates fortified at a higher level, corresponding to 10X the limit of quantitation, 300 ppt. For each analyte, the two mass transitions described above were evaluated.

2.6 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 control matrix against calibration standard solutions prepared with water. The matrix-matched standards were prepared by diluting mixed standards of each analyte with control drinking or surface water to 0.015, 0.03, and 0.06 ng/mL, equivalent to 0.5x, 1x, and 2x the LOQ, respectively.

The data generated were evaluated by comparing the average area response of the standards for 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%, calculated as the "Mean Area Change (%)". For each matrix/ion transition, an overall average "Mean Area Change (%)" across the three tested

concentrations was calculated to make a general assessment of acceptability with respect to matrix effects.

2.7 Stability of Extracts

As the method does not consist of a typical “extraction” – the water samples are diluted and analyzed – “extracts” and “final volume” are used interchangeably in this report. The stability of each analyte in stored “extract” solutions was determined in conjunction with the subject method validation study. The stability in the final volume, the solution prepared for LC-MS/MS injection, was established for each matrix by reanalyzing several control and recovery samples which had been stored under refrigeration at the final volume stage. Quantification of the analytes in the stored samples for this experiment was performed for the primary mass transitions.

4. CALCULATIONS AND RAW DATA

An example calculation is included in Appendix C (page 41).

5. STATISTICS AND DATA INTEGRITY

Statistical treatment of the data included simple descriptive statistics, such as determinations of averages, standard deviation and/or RSD for the procedural recoveries and area counts and calculation of the calibration curve and correlation coefficient (r) by linear regression of the instrument responses for the reference standards. The statistical calculations throughout this report were performed using an automated computer spreadsheet (Microsoft Excel®) and were rounded for presentation purposes. Slight differences may be noted in hand calculations using the recoveries presented in the tables. These are due to rounding and have no effect on the scientific conclusions presented in this report. The detailed analytical data may be consulted for confirmation of the calculated results.

Several measures were taken to ensure the quality of the study results. The quality assurance unit at BASF inspected the analytical procedures for compliance with Good Laboratory Practices that included adherence to the protocol. The dates inspected are detailed in the quality assurance unit statement. Study samples and test and reference items were maintained in secured (i.e. pad-locked) storage with limited access. Freezer and refrigerator temperatures were continuously monitored by electronic means.

6. SUMMARY OF METHOD

Summaries of the method parameters and characteristics are provided in Table 5 and Table 6.

7. INDEPENDENT LABORATORY VALIDATION

The method was the subject of a successful independent laboratory validation, completed on the first trial (attempt) for each water matrix (surface and drinking water) and the LC-MS/MS ion transitions (primary and secondary) available for the method, using matrix-matched standards (Reference 1). The ILV study report stated that “[t]he method is well-written and contains a fair amount of comments to guide the analyst through the procedure for the first time.

The ILV report also documented the following changes performed to the method as written: A Waters Acquity HPLC HSS T3 column with slightly different dimensions (2.1 x 100 mm, 1.8 µm) was used and the mobile phase gradient for both water types (%phase A, acidified water, is shown here for brevity) was modified to the following: at 0.00 minutes (85%), 0.02 (85.0%); 0.40 (60%); 2.20 (35%); 3.80 (10%); 5.50 (10%); 6.5 (85%); and 10.00 minutes (85%).

9. CONCLUSIONS

The results of this method validation study demonstrate that BASF Analytical Method No D1612/01 fulfils the requirements with regard to specificity, repeatability, limit of quantification, and recoveries and is, therefore, applicable to correctly determine residues of the fungicide triticonazole in water.

10. PROTOCOL, AMENDMENTS, AND DEVIATIONS

The study was conducted according to a study protocol. All protocol deviations that occurred during the conduct of this study were reported and reviewed by the Study Director. A list of all protocol amendments and deviations is provided in Appendix M. None of the changes had an impact on the validity of the study.

11. REFERENCES

1. Budgeon, A. D. (2017) Independent Laboratory Validation of BASF Analytical Method D1612/01: “Method for the determination of BAS 595 F (Reg.No. 4378513) and Its Z-isomer (Reg.No. 5079359) in Surface and Drinking Water by LC-MS/MS". BASF Study Number 808757. BASF Reg. Doc. No. 2016/7011310.

Table 2. Influence of Matrix on LC/MS/MS Response

Analyte	Standard Conc. (ng/mL)	Mean % Difference in Area Count[1]			
		Surface Water, 1º Ion Trans.	Surface Water, 2º Ion Trans.	Drinking Water, 1º Ion Trans.	Drinking Water, 2º Ion Trans.
Triticonazole (E-isomer)	0.015	3	2	12	18
	0.03	5	7	17	16
	0.06	2	0	10	12
	Overall Mean:[2]	**3**	**3**	**13**	**15**
Triticonazole (Z-isomer)	0.015	5	6	19	18
	0.03	4	1	15	12
	0.06	3	2	15	14
	Overall Mean:[2]	**4**	**3**	**16**	**15**

1. Mean percent area count difference between matrix-matched standards and solvent-based standards, at three standard concentration levels.
2. Overall mean ("Mean Area Change (%)") calculated using the absolute value of the result for each concentration level.

Table 3. Storage Stability of Triticonazole in Standard Solutions

Analyte	Standard Tested	Solvent / Conditions[1]	Limit of Demonstrated Storage Stability[2]
Triticonazole (Parent)	Intermediate/Fortification[3]	Methanol	4 months (127 days)
	Calibration	Water	4 months (121 days)
Triticonazole (Z-isomer)	Intermediate/Fortification[3]	Methanol	3 months (103 days)
	Calibration	Water	3 months (103 days)

1. Each stored under refrigeration in the dark in amber glass bottles.
2. The stability criteria: average concentration ±20% of nominal, based on LC/MS/MS analysis.
3. The stock solutions (typically 1 mg/mL) are also prepared in methanol.

Table 4. Storage Stability of Triticonazole in Extracts

Analyte	Solution Tested[1]	Limit of Demonstrated Storage Stability (days)
Triticonazole (parent and Z-isomer)	"Extract"	Surface water, 16 days; Drinking water, 14 days

1. Samples were stored under refrigeration prior to re-analysis.

Table 5. Summary Parameters for the Analytical Method Used for the Quantitation of Residues of Triticonazole in Water

Method ID	BASF Analytical Method No. D1612/01
Analyte(s)	Residues of triticonazole and its Z-isomer in water
Extraction solvent/technique	None. Residues of triticonazole in water samples (10 mL each) are analyzed by direct injection after filtration.
Cleanup strategies	Filtration (0.45 µm PTFE syringe filter)
Instrument/Detector	Liquid chromatography (LC) positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) monitoring ion transitions at m/z 318→70 (proposed as the primary transition for quantitation) and m/z 320→70 (typically for confirmatory purposes) for both isomers. The isomers are separated by their retention times on the LC column, enabling quantitation of the contribution of each analyte. All analyses are performed on an ultra-HPLC system (considered synonymous with "HPLC" for the purposes of this study report) using an XSelect HSS T3 C18 column (2.1 X 150 mm, 2.5 µm particle size) and mobile phase gradient water:methanol, each acidified with 0.1% formic acid (flow rate 600 uL/minute).
Standardization method	Direct comparison of the sample peak responses to those of external standards
Stability of std solutions	The stability of the analytes, triticonazole and its Z-isomer, in standard solutions has been determined. Each analyte has been shown to be stable in standard solutions prepared in methanol and in calibration standards prepared in water by serial dilution of intermediate/foritifcation standards (also prepared in methanol) for at least 3-4 months when stored under refrigeration.
Retention times (Approximate, expected retention times)	Parent triticonazole, 4.95 minutes Z-isomer of triticonazole, 5.01 minutes

Table 6. Characteristics for the Analytical Method Used for the Quantitation of Residues of Triticonazole in Water Matrices

Analyte	Residues of triticonazole and its Z-isomer in water
Equipment ID	Acquity ultra-HPLC chromatographic system with a XSelect HSS T3 C18 column (2.1 X 150 mm, 2.5 µm) is used with a mobile phase gradient of acidified water and acidified methanol (85:15 to 5:95, v/v, over ~5.5 minutes, flow rate 600 uL/minute).
Limit of quantitation (LOQ)	The validated LOQ for residues of triticonazole in water is 30 ppt for each analyte.
Limit of detection (LOD)	The LOD was set at 20% of the LOQ, or 6 ppt.
Reliability of the Method/ [ILV]	A successful independent laboratory validation [ILV] has been conducted for BASF Analytical Method No. D1612/01 for the determination of residues of triticonazole in water. The values obtained are indicative of the reliability of Method No. D1612/01.
Linearity	The method-detector response was linear over the 0.006 to 0.6 ng/mL range for the analysis of each analyte in water (both transitions) for the method validation sets.
Specificity/ Selectivity	The control chromatograms generally have no peaks above the chromatographic background and the spiked sample chromatograms contain only the analyte peak of interest. Peaks were well-defined and symmetrical. There appeared to be no carryover to the following chromatograms.
	An 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%).
Confirmatory technique	Due to the high selectivity and specificity of LC-MS/MS an additional confirmatory technique is not necessary.
Time required	A set of 13 samples (one reagent blank, two controls, and 10 fortified samples for recovery experiments) requires about 8 hours of work (calculation of the results included).

Appendix B. Procedure used for Method Validation

Correction:

The HPLC column catalogue number is 1860067<u>3</u>9 not 1860067<u>3</u>7 as shown below (p. 3 of 11).

ABSTRACT

BASF Method D1612/01 is developed to determine the residues of BAS 595 F and its Z-isomer in surface and drinking water using LC-MS/MS at BASF Crop Protection, Research Triangle Park, N.C.

Short description of the method:

A 10 mL (or 10 g) water sample is measured and filtered. The sample is then ready for analysis using LC-MS/MS.

The method has a limit of quantitation of 30 ng/L (30 ppt) in water for each analyte. The limit of detection in water for each analyte is 6 ng/L.

1 INTRODUCTION

BAS 595 F or triticonazole is a fungicide used against several diseases in various crops. The analytical method D1612/01 offers the possibility to determine residues of BAS 595 F and its Z-isomer in water. Method D1612/01 was successfully tested during method development in surface and drinking water for all analytes.

2 MATERIALS

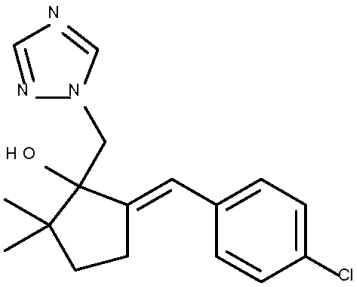
2.1 Safety

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. Store work clothing 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 Safety Data Sheets (SDS) 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.

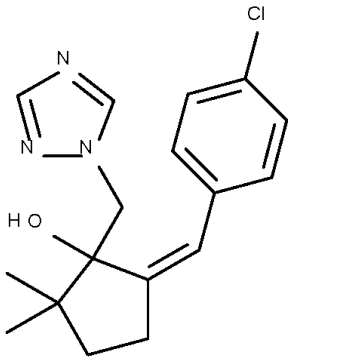
2.2 Test and Reference Items

Test and reference items should be stored according to the information provided in the certificate of analysis.

Internal-Code	BAS 595 F
Common Name	-----
IUPAC Name	(RS)-(5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol
BASF Reg. No.	4378513
CAS-No.	138182-18-0
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8



Internal-Code	M595F014 (Z-isomer)
Common Name	----
IUPAC Name	(1RS)-(5Z)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol
BASF Reg. No.	5079359
CAS-No.	-----
Molecular Formula	$C_{17}H_{20}ClN_3O$
Molecular Weight	317.8



2.3 Equipment:

Equipment	Size, Description	Manufacturer	Catalog No.
Balance, Analytical	Model AT100	Mettler	----
Beakers	Various Sizes	PYREX Brand, VWR Scientific Products	13922-029
Pasteur pipettes	Glass, disposable, 5 ¾"	VWR	14673-010
Bottle, Amber Glass with Teflon-lined caps	Various sizes	VWR	-----
Culture Tubes	Glass,disposable, 16x100mm size	Fisher	14-961-29
Culture tube caps	16 mm	VWR	60828-768
HPLC Column Xselect HSS T3 C18	150 x 2.1 mm, 2.5 µm particle size	Waters	186006737
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 6500	AB Sciex	---
Syringe, Disposable	1 mL	VWR	
Syringe filter	0.45 µm PTFE	Pall Gelman	---
Various Flask, Volumetric	100, 50, 25 ,10 and 5 mL	Various	---
Volumetric pipettes	Various sizes	VWR	---
Vortex mixer	Genie 2	VWR	58816-121

Note: The equipment and instrumentation listed above represents typical laboratory equipment and can be substituted by equipment of similar technical specifications. Suitability of the entire set of equipment is confirmed if the recoveries of the fortification experiments are in the expected concentration range.

2.4 Reagents

2.4.1 Chemicals:

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Methanol	HPLC Grade	EMD	MX0475P-1
Water, e.g. Baker® or Millipore®	Gradient Grade	BDH ARISTAR PLUS	87003-652
Formic Acid	Reagent Grade	Sigma-Aldrich	F0507-100mL

Note: Equivalent reagents and chemicals from other suppliers may be used. If not stated otherwise, common laboratory grade chemicals are used.

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.

Note: The total volume of solutions / mixtures prepared can vary depending on the required total amounts; however, mixture ratios have to be kept as described. If necessary, the solutions may also be prepared in different volumes as long as the proportions are not modified.

2.4.3 Standard Solutions

Stock Solution

Prepare a 1.0 mg/mL stock solution individually by weighing an appropriate amount of each analyte into a flask and add the required volume of methanol.
For example, weigh 10 mg of BAS 595 F into a 10-mL volumetric flask. Dissolve and dilute to mark with methanol. This creates a solution containing 1 mg/mL of BAS 595 F. Ensure a complete homogeneous solution (e.g., by sonication and/or vortexing).
Standard calibration and fortification solutions should initially be confirmed to show correct preparation of the solutions. This can be achieved by 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 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 for BAS 595 F and its Z-isomer.

Take solution (µg/mL)	Aliquot Volume (mL)	Dilute with methanol to a final volume of (mL)	Final Concentration (µg/mL)
1000	0.1 (of each solution)	10	10
10	1	100	0.1
0.1	1	10	0.01

Note: Different concentration schemes can be used, if different fortification levels are required.

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. Prepare the calibration standards as exemplified in the table below and ensure a complete homogeneous solution (e.g. by sonication or vortexing).

Preparation of standard solutions for calibration for BAS 595 F and its Z-isomer.

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with water to a final volume of (mL)	Final Concentration (ng/mL)
*100	3	50	6*
6	10	100	0.6
6	2.5	100	0.15
6	1.0	100	0.06
6	0.5	100	0.03
6	0.1	100	0.006

* Not intended to be a calibration standard but needed to prepare subsequent calibration solutions.

Note: Different concentration schemes can be used, if different calibration levels are required.

Matrix-matched standards are required for this method when determining residues in surface and drinking water matrices. Standard solutions may be used for evaluation of matrix-effects.

Depending on the matrix, significant matrix effects may interfere with the analysis of the samples. If significant matrix-effects occur, matrix-matched standards may be utilized. Matrix-matched calibration standards are used for quantitation when signal suppression or enhancement is >20% compared to the response for standards prepared in calibration solution alone. In this case, calibration standard solutions are prepared in matrix solution, i.e., using a final volume mixture from multiple control samples or using a large batch of sample, carried through the analytical procedure. Matrix-matched standards should be prepared in a way that the matrix load is at least 90% of the matrix load in the unknown samples. See section 3.5 for details on matrix-matched standards preparation.

2.4.4 Stability of Standard Solutions

BASF recommends that stock solutions (1 mg/mL) in methanol be prepared freshly every 3 months. Dilutions of stock solutions should be stored refrigerated according to their established storage stability in the particular solvent.

The stability of BAS 595 F and its Z-isomer in stock, fortification and calibration solutions will be established during the method validation if needed.

3 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Sample homogenization is not needed for water samples.

3.2 Sample Storage

Water samples are stored frozen in clean amber Nalgene (HDPE) bottles.

3.3 Weighing and Preparation of Fortified /Treated Samples

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 vortex for approximately 1 min, inverting the tube several times during the vortexing to ensure homogenization.

The following scheme may be used:

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification
Control	10 g (or mL)	-	-	0.00 ng/L
Fortification (**LOQ**)	10 g (or mL)	10 ng/mL	0.03 mL	30 ng/L * (30 ppt)
Fortification (**10xLOQ**)	10 g (or mL)	100 ng/mL	0.03 mL	300 ng/L (300 ppt)
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 should not exceed 1% of sample volume.

3.4 Preparation for Measurement

Filter the samples through a syringe filter with a 13 mm, 0.45µm PTFE filter.

3.5 Influence of Matrix Effects on Analysis

Depending on the matrix, significant matrix effects may interfere with the analysis of the samples. If significant matrix-effects occur, matrix-matched standards may be utilized. During method development, matrix effects were observed and matrix matched standards were needed.

a) Prepare precursor standard solutions for matrix-matched calibration standards according to the following table:

Preparation of Precursor Solutions for Matrix-Matched Standards for BAS 595 F and Its Z-isomer.

Initial Concentration (ng/mL)	Aliquot Volume (mL)	Dilute with HPLC water to a final volume of (mL)	Final Concentration (ng/mL)
10,000	0.6	100	60
10,000	0.15	100	15
100	6	100	6
100	3	100	3
6	10	100	0.6

b) When preparing 5 matrix-matched standards, prepare at least two extra control samples by completing all steps through 3.4.
c) Combine all samples prepared according to 3.5 [b] above into one sample container and vortex to ensure homogeneity.
d) Prepare matrix-matched calibration standards according to the table below using precursor standard solutions prepared in 3.5 [a] and control matrix in 3.5 [c]:

Preparation of Matrix-Matched Standards for BAS 595 F and Its Z-isomer.

Take Precursor Solution (ng/mL)	Volume of Precursor Solution (mL)	Dilute with Control Matrix to a final volume of (mL)	Final Concentration (ng/mL)
60	0.01	1	0.6
15	0.01	1	0.15
6	0.01	1	0.06
3	0.01	1	0.03
0.6	0.01	1	0.006

e) Dilute high residue samples to an appropriate concentration with filtered control matrix prepared in 3.5 [c].

3.6 Stability in Sample Matrix

Stability in surface and well water will be tested during the method validation.

4 QUANTIFICATION AND CALCULATION

4.1 Set-up of the analytical run

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

Reagent blanks or blanks can also be injected if considered 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 at least be injected twice. At least 5 calibration levels are needed.

4.2 Instrumental analysis

4.2.1 Instrumentation and Conditions

Reg. No.'s 4378513 and 5079359	Parameter		
Chromatographic System	Waters Acquity UPLC System		
Analytical-column	XSelect HSS T3 C18, 2.1 X 150 mm, 2.5µm particle size		
Column Temperature	50°C		
Injection Volume	20 µL		
Mobile Phase A Mobile Phase B	Water / formic acid, 1000/1, v/v Methanol / formic acid, 1000/1, v/v		
Flow Rate	600 µL/min		
Gradient (including wash and equilibration)	Time (min)	Phase A	Phase B
	0.00	85	15
	0.02	85	15
	0.63	60	40
	3.35	35	65
	4.10	5	95
	5.45	5	95
	5.50	85	15
	6.00	85	15
Detection System	AB Sciex API 6500 Mass Spectrometer		
Ionisation	Turbo Spray (ESI) Source Temp.: 550°C		
Analyte	**Transitions**	**Polarity**	**Expected Retention Time**
Reg. No. 4378513 **BAS 595 F E**	318 --> 70* 320 --> 70	positive	Approx 4.95 min
Reg. No. 5079359 **BAS 595 F Z**	318 --> 70* 320 --> 70	positive	approx. 5.01 min.

*proposed as quantification transition. Any of these transitions could be used for quantitation in case interference is observed at the same retention time

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

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 standards for LC-MS/MS in the range of 0.6 ng/mL to 0.006 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 g (or 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 595 F in ng/L are calculated as shown in equations **I** and **II**:

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

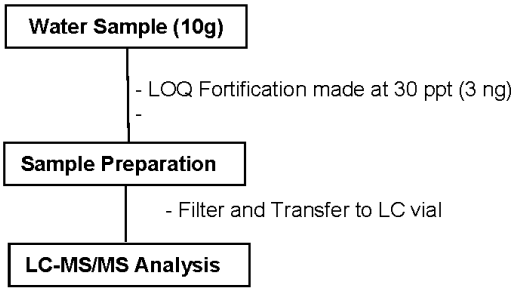
II. **Residue [ng/L]** $= \frac{V_{end} \times C_A}{G \times A_F}$

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
A_F = Aliquot factor (1 for this method)

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

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

5 FLOWCHART



6 METHOD MANAGEMENT AND TIME REQUIREMENTS

The analysis of one series of samples (= 13 total samples, 1 reagent blank, 2 controls, 5 fortified samples at LOQ and 5 fortified samples at 10x LOQ) requires 1 working day (8 hours) to complete. This time includes the calculation of the results, the preparation of the equipment as well as the reporting of all raw data under GLP.

7 CONCLUSION AND METHOD CAPABILITIES

Recoveries, Chromatograms, and Calibration Curves
Recovery data will be provided in the validation report of the analytical method D1612/01.

Limit of Quantification (LOQ) and Limit of Detection (LOD)
The limit of quantification is defined as the lowest fortification level successfully tested. The limit of quantification is 30 ng/L (30 ppt) for all analytes. The limit of detection is estimated to be 20% of the limit of quantification, equivalent to 6 ng/L for all analytes. The lowest standard for each analyte in the calibration curve has good sensitivity, hence a signal to noise ratio greater than 3:1.

Selectivity
The tested untreated surface and well water samples showed no significant interferences (<30 %) at the retention time of the analytes of interest.

Confirmatory Techniques
The HPLC-MS/MS determination for BAS 595 F and its Z-isomer is a highly selective detection technique, and quantitation is possible at two different mass transitions.

DEFINITIONS AND ACRONYMS

Sample Set:	A group of samples that are extracted and cleaned up at the same time using the same method represented.
Untreated Sample:	A sample that has not been treated with the test substance.
Control Sample:	Usually an untreated sample used for fortification experiments (can be acquired from same study or from a different source).
Unknown Sample:	The samples with unknown residues.
Treated Sample:	A sample that has been treated with the test substance.
Blank:	Solvent, solution or mobile phase injected together with a sample set.
Reagent Blank:	A complete analysis conducted using solvents and reagents only in absence of any sample. Also known as blank of reagents or procedural blank. This sample is analyzed within the sample set in order to evaluate possible contamination on chemicals/reagents.
Procedural Recovery:	A control sample to which a known amount of analyte has been added before sample work up. This sample is then carried through the method and analyzed with the unknown samples in order to determine the reliability of the method.
Instrument Recovery:	A control sample which is carried through the method and to which a known amount of analyte has been added before injection. This sample is analyzed within the sample set in order to evaluate the matrix effect in the instrument.
Analytical Run:	A group of samples that undergo a determinative measurement on an analytical instrument (such as GC, HPLC, CE, GC/MS, or LC/MS/MS) in a defined and continuous sequence under identical instrumental conditions.
Limit of Quantitation (LOQ):	Lowest tested concentration of the analyte in a sample that can be determined with acceptable accuracy and precision according to the method which is also known as reporting limit.
Limit of Detection (LOD):	Concentration of analyte equivalent to a defined percentage of the limit of quantitation of the method (e.g 20% of LOQ). At this concentration, the analyte must be qualitatively detectable in sample matrix (analyte peak height at least 3-5 x baseline noise).

Appendix C. Example Calculations

Typical Recovery Calculation for LC/MS/MS Quantitation

Sample No. 819169-1-4. Control surface water sample fortified at the LOQ with triticonazole (both analytes), Master Sheet No. 819169-01.

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

	Triticonazole (E-isomer, primary ion transition)
Peak Area =	6149
Intercept =	684
Slope =	1.66×10^5
Conc. (ng/mL) =	0.033

The concentration of analyte in ug/kg (ppb) is calculated as shown in equation:

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

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 (grams)
A_F = Aliquotation factor

	Triticonazole (E-isomer, primary ion transition)
V_{end} =	10 mL
A_F =	100%
G =	10.0 g
Conc. (ng/mL) =	0.033
Residue (ug/kg) =	0.033

Net residue (ug/kg of analyte) = Residue (ug/kg of analyte) - Residue in Control (ug/kg)

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

	Triticonazole (E-isomer, primary ion transition)
Amount fortified (ug/kg) =	0.03
Residue (ug/kg) =	0.033
Residue in control =	0.0000
%Recovery	110%

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

Appendix M. Protocol Amendments and Deviations

Protocol Amendments and Deviations

Deviation 1: The protocol stated that a volumetric pipette would be used to administer the test substances to the test system; however, as a volumetric glass pipette to deliver a 30 uL volume was not available a micropipette was used instead. In addition, the concentrations of the fortification solutions were not verified prior to being used in the subject study; however, the same solutions were used in method development just prior to use in the subject study and proved to be of accurate concentration.

None of the amendments / deviations noted above affect the validity of the study.