ABSTRACT

The objective of this study was to validate the analytical method L0199/01 for the determination of 1,2,4-Triazole (Reg. No. 87084) in water by using LC-MS/MS.

Principle of the method. A 2 mL water sample is given on a SPE-column. The column is washed with water and the received filtrate is evaporated to dryness in the nitrogen evaporator at 45°C. Subsequently, the residue is dissolved in 0.5 mL water. The concentration of 1,2,4-Triazole is measured by HPLC-MS/MS.

Test conditions. The method was validated at two fortification levels (0.05 and 0.5 µg/kg) for two water systems (surface water and ground water). For each fortification level and matrix, five replicates were analysed. Additionally, at least two replicates of unfortified samples were examined. One mass transition was evaluated for the test item and one for the internal standard. The results were confirmed by the use of two different HPLC columns.

Limit of Quantification (LOQ) and Limit of Detection (LOD). The limit of quantification (LOQ) was defined by the lowest fortification level successfully tested. LOQ is 0.05 µg/kg for 1,2,4-Triazole, which corresponds to a concentration in the filtrates of 0.2 ng/mL. The limit of detection (LOD) was estimated as 25% of the limit of quantification; equivalent to 0.013 µg/kg (corresponds to 0.05 ng/mL).

Selectivity. The method L0199/01 determines 1,2,4-Triazole in water. Significant interferences (> 30% LOQ) were not observed in unfortified samples at the retention time of the analytes using the primary chromatographic separation (Aquasil C18 column).

1 INTRODUCTION

1.1 Scope of the Method

The objective of this validation study was to validate the method L0199/01for the determination of 1,2,4-Triazole (Reg. No. 87084) in water by LC-MS/MS.

1,2,4-Triazole is a metabolite of various pesticides. Therefore, a residue analytical method for the detection and quantification of 1,2,4-Triazole in surface and ground water was needed for monitoring purposes with a limit of quantification (LOQ) of 0.05 µg/kg.

As described below, the BASF Method No. L0199/01 allows the determination of the analytes with the required limit of quantification in surface and ground water. This method was developed at BASF SE, located in Limburgerhof (Germany). To demonstrate the validity of the method, recovery trials with spiked water samples were performed.

The method was validated at two fortification levels (LOQ and 10x LOQ) for surface water and ground water.

1.2 Principle of the Method

A 2 mL water sample is given on a SPE-column. The column is washed with water and the received filtrate is evaporated to dryness in the nitrogen evaporator at 45°C. Subsequently, the residue is dissolved in 0.5 mL water. The concentration of 1,2,4-Triazole is measured by HPLC-MS/MS.

2 MATERIALS AND METHODS

2.1 Test systems

The following test systems were considered in this study of validation:

Test System 1: **Surface water**

Test System 2: **Ground water**

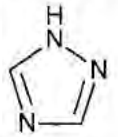
Test System 3: **Ultra Pure Water**

The description and characterization of the surface and groundwater samples are given in the respective attached certificates (Figure A.26 and Figure A.27)

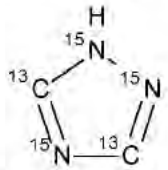
2.2 Test Item and Internal Standard

The certificates of analysis for the test item and the internal standard are shown in Figure A.28 and Figure A.29.

2.2.1 1,2,4-Triazole

Registry No.:	87084
CAS No.:	288-88-0
Chemical name (IUPAC):	1,2,4-(1H)-triazole
Structural formula:	
Molecular formula:	$C_2H_3N_3$
Molecular mass:	69.1 g/mol
Batch No.:	AC10194-134
Purity:	99 %
Test substance type	ME
Storage advice	keep in refrigerator (approx. +4°C) or cooler
GLP	yes
Expiration date	01.04.2022

2.2.2 1,2,4-Triazole (stable isotope labelled, Internal Standard)

Registry No.:	87084
CAS No.:	288-88-0
Chemical name (IUPAC):	1,2,4-(1H)-triazole
Structural formula:	
Label	3,5-C13;1,2,4-N15
Molecular formula:	$C_2H_3N_3$
Molecular mass:	69.0667 g/mol
Batch No.:	992-1005
Chemical Purity:	98.4 %
Storage advice	at low temperature and in the dark
GLP	yes

2.2.3 Stability of the Test Item

2.2.3.1 Stability of Calibration Standard Solution

The stability of 1,2,4-Triazole in the standard solution (1 ng/mL), prepared in water, was investigated within this study.

For this purpose, the standard solution was stored at 4 °C for 7, 16 and 30 days (filtrated after 7, 14 and 30 days). At each sampling time point, the concentration of 1,2,4-Triazole was measured against freshly prepared standards within one analytical queue.

Quantification of the analyte was done for one mass transition and by using an internal standard. Hypercarb HPLC column was used in the analysis. Recovery data are presented in the results (section 3.2).

2.2.3.2 Stability of Extracts

Additional to the storage stability of the standard solution, the stability of 1,2,4-Triazole in the filtrates obtained from fortified ground water samples was investigated at fortification level 0.00025 mg/kg.

Data were obtained from the stored extracts at day 0 (starting values) and after 7 days at 4°C.
At both sampling time points, the concentration of 1,2,4-Triazole was measured against freshly prepared standards within one analytical queue.

For quantification an internal standard was used. Analysis was performed by using an Hypercarb HPLC column. Recovery data are presented in the results (section 3.3).

2.3 Materials and Methods

2.3.1 Equipment

Equipment	Size, Description	Manufacturer	Catalog No.
Balance	Analytical, AT 261	Mettler, Giessen (Germany)	--
Balance	Precision balance	Sartorius LP 5200 P	--
Pipette	1000 µL, 100 µL, 10 µL	Gilson Medical Electronics S.A., F 95400 Villierle-Bel, France	F148506 F148504 F148503
Tubes	12 mL	Schott Glaswerke, Mainz	
Autosampler vials	0.2 mL, N 11 with integ. Inset	Macherey-Nagel GmbH, D 52313 Düren	702709
Snap caps	Snap Ring Caps N11 with cross-slit	Macherey-Nagel GmbH, D 52313 Düren	702717.2
Ultrasonic bath	2 liters	Elma. Transsonic 460	--
SPE-column	Strata-X-CW 3 ml 60 mg, 33 µ	Phenomenex Aschaffenburg	8B-S035-UBJ
Nitrogen-Dryer	1-VIS (N-Vap)	VLM	--
Regular laboratory equipment	--	--	--

2.3.2 Reagents

2.3.2.1 Chemicals

Chemical	Grade	Manufacturer/Supplier	Catalog No.
Water, e.g. Baker® or Millipore®	Gradient Grade	J.T.Baker / Millipore/Waters	--
Methanol	Gradient Grade	Merck	1.06018

2.3.2.2 Solutions and Solvent Mixtures

Description	Code	Composition
Solution 1	S1	Water, e.g. Baker® or Millipore® (Ultra Pure Water)
Solution 2	S2	Methanol
Internal standard solution	IS	1 ng/mL 1,2,4-Triazole (isotopic labelled) in water
HPLC mobile phase A	LC1	1% Formic Acid in Water Add 1000 mL of water and 10 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	1% Formic Acid in Methanol Add 1000 mL of methanol and 10 mL of concentrated formic acid into a, e.g., 1L Erlenmeyer flask and mix well to ensure complete homogeneous solution.

2.3.2.3 Standard Solutions

Stock Solutions

A 1 mg/mL stock solution was prepared by weighing an appropriate amount of the test item into a flask and adding the required volume of water (S1).

An internal standard stock solution was prepared accordingly with a concentration of 1 mg/mL. The stock solution was diluted volumetrically as described in the following table and mixed to ensure a complete homogeneous solution.

Preparation of Internal Standard Solutions (IS)

Take solution (µg/mL)	Volume (mL)	Dilute with S1 to final volume of (mL)	Concentration
1000 (Stock)	1	10	100 µg/mL
100	1	10	10 µg/mL
10	1	10	1 µg/mL
0.25	1	250	**1 ng/mL**

Fortification Solutions (dissolved in water)

Standard solutions for fortification were prepared by dilution with water (S1) as exemplified in the table below. Sonication or vortexing were considered for ensuring a complete homogeneous solution.

Preparation of Fortification solutions

Take solution (µg/mL)	Volume (mL)	Dilute with S1 to final volume of (mL)	Concentration
1000 (Stock)	1	10	100 µg/mL
100	1	10	10 µg/mL
10	1	10	1 µg/mL
1	1	10	0.1 µg/mL
0.1	1	10	**0.01 µg/mL**
0.01	1	10	**0.001 µg/mL**

Calibration Standard Solutions (dissolved in internal standard solution)

Standard calibration solutions for LC-MS/MS analysis were prepared from the stock solution of the test item by dilution with the internal standard solution (1 ng/mL) as needed. The solutions were made up as follows:

Preparation of standard solutions for calibration

Take solution (µg/mL)	Volume (mL)	Dilute with IS to final volume of (mL)	Concentration
1000 (Stock)	1	10	100 µg/mL
100	1	10	10 µg/mL
10	1	10	1 µg/mL
1	1	10	0.1 µg/mL
0.1	1	10	0.01 µg/mL
0.01	5.00	10	5.00 ng/mL
0.01	2.50	10	2.50 ng/mL
0.01	1.00	10	1.00 ng/mL
0.01	0.50	10	0.50 ng/mL
0.01	0.25	10	0.25 ng/mL
0.01	0.10	10	0.10 ng/mL
0.01	0.05	10	0.05 ng/mL

During method development, it was found that calibration solutions were stable (less than 10% decline) for at least 30 days when stored refrigerated. Therefore, stock solutions (1 mg/mL) in water were made fresh every three month. Dilutions of stock solutions in water were also kept in the refrigerator for no longer than one month.

2.3.3 Analytical Procedure

2.3.3.1 Weighing and Fortification

For fortification, 2 g control water samples were fortified with the spiking solution as described in the following table (2 g corresponded to a volume of 2 mL since a density of 1.00 g/cm³ was assumed):

Sample Type	Sample Weight	Concentration of Spiking Solution	Volume of Spiking Solution	Level of Fortification	Internal Standard [1 ng/mL]
Control	2 g	-	-	0.00 µg/kg	0.5 mL
Fortification (LOQ)	2 g	1 ng/mL	0.1 mL	0.05 µg/kg *	0.5 mL
Fortification (10xLOQ)	2 g	10 ng/mL	0.1 mL	0.50 µg/kg	0.5 mL

* Limit of quantification (LOQ)

2.3.3.2 Sample Work-Up

An SPE-column was pre-conditioned with 1 x 2 mL methanol (S2) followed by 1 x 2 mL water (S1). These solutions were discarded. The bottom valve must be closed after conditioning.

For treated samples and control samples, 2 ml water sample and 0.5 mL internal standard solution (1 ng/ml) were given on the SPE-column and mixed directly in the column. For fortifications, the appropriate volume of spiking solution was added as well and thoroughly mixed with the water in the SPE-column.

Then the bottom valve was opened and the volume passes with one drop per two seconds. Weak vacuum was applied to assure the desired flow rate. The filtrate was collected in a 12 mL tube and was used directly for further analysis. The filtrate was concentrated to dryness in the nitrogen evaporator at 45°C and the residue was dissolved in 0.5 mL water. SPE-column was washed with 3 x 0.5 mL water (S1).

By the sample processing, a small blank value is obtained (probably from the SPE-column). This blank value is smaller than the LOD of the method (APL004, data not shown).

2.3.3.3 Preparation for Measurement

In the case of concentrations in the range of the LOQ, no further dilutions were necessary (final volume of 0.5 mL).

2.3.3.4 Influence of Matrix effects on Analysis

The method L0199/01 used an internal standard (stable isotope labelled 1,2,4-Triazole) for quantification. Any influence by the matrix carried with the samples affected the analyte 1,2,4-Triazole as well as the internal standard in the same way. Therefore, the matrix does not influence the analytical results.

2.3.4 Set-up of the Analytical Run

Reagent blanks or blanks were injected as necessary. Each injection began and ended with an injection of a calibration standard. Standards were interspersed with samples. Each calibration standard was at least injected twice. Seven calibration levels were injected.

2.4 Instrumental Analysis

2.4.1 Instrumentation and Conditions

	Parameter			
Chromatographic System	Agilent HP 1100 with CTC Autosampler			
Analytical columns	Thermo Aquasil C18 (3 µm, 150 mm L x 3 mm I.D.)			
	Thermo Hypercarb (3 µm, 50 mm L x 4.6 mm I.D.) *			
Column Temperature	20-25°C (RT)			
Injection Volume	50 µL			
Mobile Phase A Mobile Phase B	Water / formic acid 100/1, v/v Methanol / formic acid 100/1, v/v			
Gradient (including wash and equilibration)	Time [min]	Phase A [%]	Phase B [%]	Flow rate [µl/min]
	0.0	95	5	800
	2.0	95	5	800
	2.1	95	5	900
	2.5	15	85	900
	5.0	15	85	900
	5.1	95	5	900
	5.2	95	5	800
	7.0	95	5	800
Detection System	PE Sciex API 4000 Mass Spectrometer			
Ionisation	Electrospray (ESI)			
Analyte	**Transitions**		**Polarity**	**Expected Retention Time**
1,2,4-Triazole	70→43		positive	approx. 1.6 min
IS	75→46		positive	approx. 1.6 min

* used for confirmatory purpose

2.4.2 Calibration Procedures

Calculation of results was based on peak area measurements using a calibration curve with internal standard. Seven calibration levels were injected. The calibration curve was obtained by direct injection of 1,2,4-Triazole standard solutions in the range of 0.05 ng/mL to 5.0 ng/mL. In all injection runs, the same injection volume was used for all samples and standards. Linear calibration functions were used for evaluation.

2.5 Calculations

2.5.1 Rounding of Decimal Places

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than were used in the actual calculation to increase readability and to indicate the approximate precision of the reported results. Minor differences in the results obtained with such "rounded" values in comparison to those obtained with higher precision values are well within the limits of the experimental accuracy and therefore of no practical concern.

2.5.2 Calculation of Residues and Recoveries

For the procedural recoveries, the sample weight was considered 2 g in the final calculation of residues [mg/kg]. The method requires also that the sample weight was 2 ± 0.05 g for fortification samples. The calculation of results is based on peak area measurements.

I. **Nominal Concentration [ng/mL]** is the concentration theoretically expected in the final volume and is calculated as follows:

$$\text{Nominal Concentration [ng/mL]} = \frac{\text{Fortification level (mg/kg)} \times G}{\text{Final Volume}(V_F)} \times 1000$$

II. **Concentration Final Volume [ng/mL]**

$$C_{A\ \text{Sample}} = \frac{\left(\frac{\text{Response Analyte}}{\text{Response IS}}\right) - \text{Intercept}}{\text{Slope}}$$

III. **Residues in the Sample Matrix [mg/kg]**

$$= \frac{V_F \times C_A}{G \times A_F}$$

V_F	=	Final volume of the extract after all dilution steps [mL]
C_A	=	Concentration of analyte obtained from the calibration curve [ng/mL]
G	=	Weight of the sample extracted [g]
A_F	=	Aliquotation factor = 1.0 (=100%)
1000	=	Factor remaining after all unit conversions

Recovery is the percentage of the fortified amount (µg or ng), which is recovered through the method. The recoveries of spiked compounds are calculated according to equation **IV**. The **Recovery corrected** was calculated by subtracting the control mean values from the analyte concentrations according to equation **V**.

IV. **Recovery (%)**

$$= \frac{\text{Residue in fortified sample} \times 100}{\text{Fortification level}}$$

V. **Recovery corrected (%)**

$$= \frac{(\text{Residue in fortified sample} - \text{Residue in control}) \times 100}{\text{Fortification level}}$$

Example:

Sample of 1,2,4-Triazole (Mass transition 70→43) in Ground Water fortified at 0.05 µg/kg (AP L001):

The following values were used in this calculation:

Worklist no.	*2012vwt0024*
Peak area of fortified sample (ForL0002)	*8638.0*
Peak area of internal standard (ForL0002)	*29035.188*
Concentration Final Volume of Control (ConL0001 / ConL0002)[1]	*0.148 ng/mL*
Slope	*0.816*
Intercept	*0.00566*
Sample Weight (G)	*2 g*
Final Volume (V_F)	*0.5 mL*
Aliquotation Factor A_F	*1.0 (= 100%)*
Conversion Factor ng → µg	*1000*

[1] *Mean area of two control samples in the same worklist*

Concentration Final Volume (C_A) $$\frac{\frac{8638.0}{29035.188} - 0.00566}{0.816} = 0.358\ ng/mL$$

Residue (Fortified Sample) $$= \frac{0.5\ mL \times 0.358\ ng/mL}{2\ g \times 1} = 0.0895\ \mu g/kg$$

Residue (Control Sample) $$= \frac{0.5\ mL \times 0.148\ ng/mL}{2\ g \times 1} = 0.0370\ \mu g/kg$$

Recovery [%] $$= \frac{0.0895\ mg/kg}{0.05\ mg/kg} \times 100 = 179\ \%$$

Recovery corrected [%] $$= \frac{(0.0895\ mg/kg - 0.0370\ mg/kg) \times 100}{0.05\ mg/kg} = 105\%$$

3.4 Summary of the Method

Type of method:	LC-MS/MS
Test systems:	Surface water (Figure A.26) Ground water (Figure A.27)

Analytes and selected mass transitions:

1,2,4-Triazole (unlabelled)	70→43
Stable isotope labelled 1,2,4-Triazole (IS)	75→46

Analytical procedure:	SPE-filtration of the water samples followed by LC-MS/MS analysis of the concentrated filtrates
Confirmatory technique:	To confirm the determination of 1,2,4-Triazole, the validation was successfully performed with two different chromatographic columns. An internal standard was used for quantification. Recovery data is reported for both columns and both matrices (Table A.5 to Table A.8).
Limit of detection (LOD):	0.013 µg/kg, corresponding to a concentration of 0.05 ng/mL in the extract
Limit of quantification (LOQ):	0.05 µg/kg (lowest fortification level), corresponding to a concentration of 0.2 ng/mL in the extract
Levels of fortification:	0.05 µg/kg and 0.5 µg/kg of 1,2,4-Triazole
Time required:	a set of 12 samples requires about 12 hours of work (calculation of the results included)

Appendix 6.2: Additional Information on the Method

Figure A.3: Method Flowchart