7. Summary

OBJECTIVE

The objective of this study was to validate as independent laboratory validation (ILV) a method for determination of 1,2,4-Triazole in water by LC-MS-MS.
This method is based on BASF SE method L0199/01 reported in the BASF SE Report No. 428292[1] (DocID 2012/1297158) (see Enclosure F).

ANALYTICAL PHASE

A full independent method validation on surface water and ground water was carried out by LC/MS-MS with quantification by using the following ions transitions:

Compound	Abbreviation	Precursor ion (m/z)	Product ion (m/z)
1,2,4-triazole	*T*	70	43
[$^{15}N_3$-$^{13}C_2$] 1,2,4-triazole	*TIS*	75	46

The principle of the method was based on a SPE purification with determination by LC/MS-MS on direct injection of the substrates.

The analysis was performed by using both columns Aquasil C18 and Hypercarb and confirmed.

Method validation

Specificity

The method is capable of determining 1,2,4-triazole in the presence of the two water systems (surface and ground water).

Calibration

The calibration curves of 1,2,4-triazole in water were tested in order to encompass the concentration in the final purified sample. The concentrations ranged from 0.05 µg/L to 5.019 µg/L corresponding to 0.0125 µg/kg and 1.2548 µg/kg respectively.

The calibration curves were tested with seven concentrations in duplicate injections.

[1] H. Penning, D. Schelling, M. Possienke (2013). "Validation of analytical method L0199/01 for determination of 1,2,4-Triazole (Reg. No. 87084) in water by LC-MS/MS". BASF SE study no. 428292.

LIMIT OF QUANTITATIVE DETERMINATION (LOQ) AND LIMIT OF DETECTION (LOD)

The target quantitative limit of determination (LOQ) at 0.05 µg/kg was confirmed for both waters by recovery tests.
The mean recovery results were within the range of 70-120% with a RSD below 20%.

The LOQ was 0.05 µg/kg while the LOD was 0.013 µg/kg for both ground and surface water.

CONCLUSION

The Indipendent Laboratory Validation was successful and has met the criteria reported in the Guideline Requirements.
No modification to the method has been done.

8. INTRODUCTION

Isagro GLP Test Site conducted an Independent Laboratory Validation (ILV) of the method BASF SE L0199/01 reported in the BASF SE Report No. 428292 (DocID 2012/1297158) for determination of residues of 1,2,4-triazole in surface and ground water

GUIDELINE REQUIREMENTS

The study was conducted according to the following guidelines and regulations:

- US EPA Ecological Effects Test Guideline OCSPP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation;
- Assigning values to non detected/non-quantified pesticide residues in human health food exposure assessments. Office of Pesticide Programs, U.S. Environmental Protection Agency, Washington, DC 20460 - March 23, 2000;
- OECD guidance document on pesticide residue analytical methods; ENV/JM/MONO(2007)17;
- EC guidance document for generating and reporting methods of analysis. SANCO/3029/99 rev.4, 11/07/00;
- Guidance document on residue analytical methods; SANCO/825/00 rev. 8.1, European Commission, Directorate General Health and Consumer Protection, 2010-11-16.

9. PROCEDURES

9.1 Receipt, storage and preparation

Ground water was drawn directly from a private well. Surface water was drawn from the Ticino river at Cameri (NO), in the Northern Italy geographical area. The geographical coordinates and the map of drawing area are shown in Enclosure D.

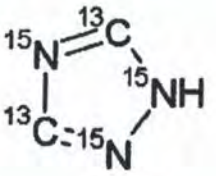
Both waters were previously characterized by the external laboratory "THEOLAB S.p.A". The water characterization is reported in the Table 1 and Enclosure B and C.

All specimens were stored in the dark from T +1 to +6 °C until the sample analysis.

At the analysis time, each sample was assigned a lab code by RA, according to the SOP.

The sample history is reported in Table 7.

9.2 Test/Reference items

	T	TIS
	1,2,4-triazole	*1,2,4-triazole-$^{15}N_3$-$^{13}C_2$*
Structural formula		
Molecular formula	$C_2H_3N_3$	$C_2H_3N_3$
Molecular weight	69.07 g/mole	74.02 g/mole
CAS number	288-88-0	unavailable
Physical state	solid	solid
Batch number	420195/1	KML-4556-2-2
Purity	99.3%	99.0%
Expiry date (dd/mm/yyyy)	10/07/2018	03/04/2017

The SS were prepared by weighing a defined amount of each reference item into a volumetric flask and making up to volume with water HPLC/MS grade.

9.2.1 Preparation of Test/Reference items solutions

Stock solutions (SS):

The SS were prepared by weighing a defined amount of each reference item into a volumetric flask and making up to volume with water HPLC/MS grade.
Weights, volumes and final concentrations are reported in the tables below:

Compound	Stock solz ID	weight (mg)	Standard purity (%)	Final volume (mL)	Final conc (mg/mL)
T	SS1	5.32	99.3	5.0	1.057
TIS	SS2	5.66	99.0	5.0	1.121

Solutions were stored in the dark in refrigerated conditions (T +1 to +6 °C) until use.

Working solutions (WS):

The WS were prepared from the stock solutions by dilution with water HPLC/MS grade as reported in the following tables.

[$^{15}N_3$-$^{13}C_2$] 1,2,4-triazole- (Internal standard)

Solz No.	Volume taken (ml)	Original Solution No.	Final volume (mL)	Final conc (µg/mL)	Final conc (ng/mL)
1	0.9	SS2	10.0	100.9	-
2	1.0	1	10.0	10.09	-
3	1.0	2	10.0	1.009	-
4	1.0	3	100.0	-	10.09
5	*5*	*4*	*50*	*-*	*1.009*

1,2,4-triazole

Solz No.	Volume taken (ml)	Original Solution No.	Final volume (mL)	Final conc (µg/mL)	Final conc (ng/mL)
6	0.95	SS1	10.0	100.4	-
7	1.0	6	10.0	10.04	-
8	1.0	7	10.0	1.004	-
9	1.0	8	10	0.1004	100.4
10	*5.0*	*9*	*50*	*-*	*10.04*
11	*1.0*	*10*	*10*	*-*	*1.004*

Each standard was pooled in a single solution in order to have a multistandard solution as reported in the following table:

Solz No.	T		TIS		Final volume (mL)	Final conc (ng/mL)	
	Volume taken (ml)	Original Solution No.	Volume taken (ml)	Original Solution No.		T	TIS
12	5.00	**10**	1	**4**	10.0	5.019	1.009
13	2.50	**10**	1	**4**	10.0	2.509	1.009
14	1.00	**10**	1	**4**	10.0	1.004	1.009
15	0.50	**10**	1	**4**	10.0	0.502	1.009
16	0.25	**10**	1	**4**	10.0	0.251	1.009
17	0.10	**10**	1	**4**	10.0	0.100	1.009
18	0.05	**10**	1	**4**	10.0	0.050	1.009

These WS were injected in order to obtain the calibration curves.

SS and WS were stored in the dark in refrigerated conditions (T +1 to +6 °C) until use.

9.3 Analytical procedure

9.3.1 Reagents

- ✓ Analytical methanol, LC-MS Chromasolv® (Sigma Aldrich);
- ✓ Formic acid 39% ACS-for analysis (Carlo Erba Reagents);
- ✓ Nitrogen gas from Nitrogen generator;
- ✓ Water, HPLC/MS grade, purified with MILLI-Q (MILLIPORE).

9.3.2 Equipment

- ✓ Analytical balance (Mettler-Toledo mod. XP105);
- ✓ Common analytical laboratory glassware and equipment for chemical laboratory;
- ✓ Dri-Block® model DB3 (Techne);
- ✓ Ultra High Performance Liquid Chromatograph equipped with degasser, binary pump, column thermostatic oven, automatic sampler and triple quadrupole mass detector (e.g.: Nexera X2 + ABSciex QTrap 6500);
- ✓ HPLC analytical column Hypercarb 100x2.1 mm 5 µm + Hypercarb pre-column 10 x 2.1 mm 5 µm (Thermo);

- ✓ HPLC analytical column Aquasil 150x3 mm + Drop-In Guards Aquasil C18 10 x 3mm 3µm (Thermo);
- ✓ SPE Strata-X-CW 3 mL, 60 mg, 33 µ (Phenomenex cod. 8D-S035-UBJ);
- ✓ Technical balance (e.g. Sartorius mod. LC 820);
- ✓ Ultrasonic bath (SONICA mod.1200 M);
- ✓ Vortex.

9.3.3 Analytical Method

9.3.3.1 Fortification

For the validation, two untreated control samples, five samples at the LOQ level and five samples at the 10xLOQ level were prepared for surface and ground water.

The substrate volume, the concentration, the volume added and the consequent spiking levels are summarized in the table below:

Sample Type	Sample Weight (g)	T		TIS		Final conc (ng/mL)	
		Volume taken (ml)	Original Solution No.	Volume taken (ml)	Original Solution No.	T	TIS
UTC	2	-	-	0.5	**5**	-	1.009
LOQ	2	0.1	**11**	0.5	**5**	0.050	1.009
10xLOQ	2	0.1	**10**	0.5	**5**	0.502	1.009

Control and fortified samples were directly injected into the LC/MS-MS after SPE purification.

9.3.3.2 Analytical Procedure

The method validation for 1,2,4-Triazole has been fully validated on ground and surface water in the GLP study BASF SE L0199/01 method.

The analytical procedure for each type of water tested is described below:

1) The Strata-X-CW cartridges (Phenomenex) were mounted in a working position on top of a vacuum manifold and conditioned with 2 mL of methanol followed by 2 mL of water. These solutions were discarded.

2) 2 mL aliquot of each water was transferred into 24 cartridges (12 for ground water and 12 for surface water).

3) 0.5 mL of internal standard solution Solz No. 5 (1 ng/mL) was added to each cartridge.

4) For fortification 0.1 mL of Solz No. 11 (1.004 ng/mL) and of Solz No. 10 (10.04 ng/mL) were added respectively into the cartridge corresponding at the LOQ level and the 10xLOQ level.
The solutions were mixed directly into the columns.

5) The mixed solutions were eluted off the cartridges under slight vacuum into a 10 mL centrifuge tubes and the SPEs were washed with 3x0.5 mL of water HPLC/MS grade.

6) The eluates were evaporated to dryness using a Dri-Block at temperature of 45°C under a gentle flow of nitrogen.

7) The remaining residue was redissolved into 0.5 mL of water HPLC/MS grade, the walls were rinsed and the solutions were sonicated for approximately 10 sec.

8) The solutions were transferred into glass vials and directly injected.

9.3.3.3 UHPLC Method

The analysis was carried out with the UHPLC/MS-MS technique.

The complete set of WS and Quality Control standards were injected in duplicate.
All the samples were injected in single injection.

The regression equation generated from the WS was used to check the linearity of response and to quantify the 1,2,4-Triazole residues in samples.

The analysis was performed by using both columns Aquasil c18 abd Hypercarby and confirmed

The operative conditions used are summarized hereafter

- *Column*: Hypercarb 100 x 2.1 mm, 5 µm
  Aquasil C18 150 x 3 mm, 3 µm

Eluent:

- **A**: (water + 1% formic acid) 95%
- **B**: (methanol + 1% formic acid) 5%

- Elution conditions:

min	%A	%B	Flow rate (mL/min)
0.0	95	5	0.800
2.0	95	5	0.800
2.1	95	5	0.900
2.5	15	85	0.900
5.0	15	85	0.900
5.1	95	5	0.900
5.2	95	5	0.800
7.0	95	5	0.800

- *Stop time*: 7 min
- *Column temp.*: 20-25 °C (room temperature)
- *Injection volume*: 50 µL

Mass spectrometric conditions

- *Interface*: Turbo Ion Spray (ESI)
- *Polarity*: positive
- *Curtain gas (CUR)* 35
- *CAD gas* High
- *IonSpray voltage (IS)* 5500 V
- *Temperature (TEM)* 550 °C
- *Gas 1 (GS1)* 45
- *Gas 2 (GS2)* 70

- *Molecular transitions and Retention time*:

Compound	Column Aquasil t_R (min)	Column Hypercarb t_R (min)	Precursor ion (m/z)	Product ion (m/z)
T	≈ 1.4	≈ 0.45	70	43
TIS	≈ 1.4	≈ 0.45	75	46

Further details on instrument settings, operative conditions and mass spectra are reported in Section 13.

9.3.3.4 Calculations

The *relative detector response*, defined as the ratio between the analyte peak area and the respective internal standard peak area, was plotted against the *relative concentration*, defined as the ratio between the analyte concentration and the internal standard concentration.

The regression equation generated by the calibration curve (type 1/x) was used to check both the linearity of response and to quantify the residue present in samples.

Peaks were integrated and the calculation of the concentration of each injected solution was performed according to the calibration curve technique.

The formula applied is:

$$R = \frac{a_{smp} - i}{s} * C_{STDIS} * \frac{V}{W}$$

where:

R: residue (in µg/kg);

a_{smp}: peak area ratio (analyte/internal std) of the sample;

s: slope of the calibration curve;

i: intercept of the calibration curve;

C_{STDIS} concentration of the internal standard added to the sample (µg/kg);

V: final volume of extract (in mL);

W: amount of the water purified (in g).

The recovery % is calculated as follows:

$$\%\ recovery = \frac{R - R_{Control}}{F} * 100\%$$

where:

R: residue found (in µg/kg);

$R_{Control}$ residue found in control samples (in µg/kg);

F: quantity of 1,2,4-triazole added in the fortified samples (in µg/kg).

For example, the residue level of 1,2,4-triazole measured in the sample 1606/1R1 (as reported in Table 4 and in Enclosure H) has been calculated as follows:

a_{smp}: T: 1723
TIS: 8945.3

ratio: 0.19262
i: 0.07414
s: 0.78768
V 0.5 mL
W: 2 g

$$R = \frac{0.19262 - 0.07414}{0.78768} \times 1.009 \times \frac{0.5}{2.0} = 0.038\ \mu g/kg$$

$$\%\ recovery = \frac{0.038 - 0.0}{0.0502} * 100 = 75.7\%$$

Correction of recoveries for trace residues in untreated samples was carried out.

Time Requirements:

The time required for the preparation and purification of 6 specimens and 25 procedural recoveries was approximately 6.2 person/hours.
The evaluation time required approximately 6.2 person/hours.
The time period from the preparation of the samples set until the completion of the data reporting was approximately 12.5 person/hours.

The UHPLC/MS-MS run was 7 minutes, 31 samples and the corresponding samples for calibration required approximately 27 instrument/hours (considering two injections for

standards and one injection for each sample. All standards and samples were analysed both with Hypercarb and Aquasil columns.

9.3.3.5 Important points

Solvent injections were interspersed often in order to purge the column and the detector.

9.4 Indipendent Method Validation

9.4.1.1 Specificity

Signals interfering potentially with the analyte at the expected retention times in the ion chromatograms were below 30 % of the LOQ (<0.05 µg/kg) in all control samples.

9.4.2 Calibration

The calibration curve was checked in a concentration range to encompass the expected range of concentrations in the final purified samples.
The calibration curve was checked before each sample sequence.

9.4.3 Accuracy

The accuracy of the method was evaluated at two different concentrations, 0.05 µg/kg and 0.50 µg/kg. Overall accuracy was calculated for the entire data set.

According to, to SANCO/3029/99 rev.4, and to OECD ENV/JM/MONO(2007)17, the set of samples was constituted by:

2 control samples	**(unfortified);**
5 samples	**at the limit of quantitative determination (LOQ);**
5 samples	**at 10 times LOQ.**

9.4.4 Precision – repeatability (r)

Precision was evaluated by calculating the %RSD at two fortification levels for both waters. Overall precision of the method was calculated for the entire data set.

9.4.5 Limit of quantitative (LOQ) and qualitative (LOD) determination

The target quantitative limit of determination (LOQ) at 0.05 µg/kg was tested for both waters by recovery tests. The LOD was 0.013 µg/kg.

9.5 Quantitative Sample Analysis

Quantitative analyses were performed according to the method described above.

Reference standard solutions were injected just before sample sequence (P suffixed samples) in order to check the chromatographic column.

The chronological complete sequences of injections and the primary data were archived with the raw data.

9.6 Graphical Software

All the graphical elaborations of data were carried out using the MultiQuant™ 3.0 and/or the EXCEL software (Microsoft Office 2013).

9.7 Measurement and data elaboration

Concentrations were automatically calculated by the integrator software MultiQuant™ 3.0.

Data elaboration was carried out by EXCEL software (Microsoft Office 2013).

The rounding of decimals, when not obtained by the above mentioned software, was executed following the F.W.Küster and A.Thiel guidelines[2].

The arithmetic means reported were automatically obtained by averaging the individual values with a greater number of decimals than usually shown in the tables.

[2] Küster and Thiel Tabelle Logaritmiche (Logarithmic Tables)–Ulrico Hoepli Editor Milan Italy.

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)

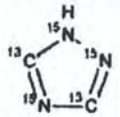
2.2 Test Item and Internal Standard

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 Villiers-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	Water / formic acid		100/1, v/v	
Mobile Phase B	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
	6.2	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 [%] $$= \frac{(0.0895\ mg/kg - 0.0370\ mg/kg) \times 100}{0.05\ mg/kg} = 105\%$$

Appendix 6.2: Additional Information on the Method

Figure A.3: Method Flowchart