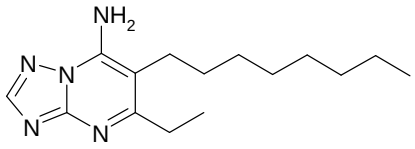
1. INTRODUCTION

Objective:

The objective of this study was to validate an analytical method for the determination of BAS 650 F in water at a limit of quantification (LOQ) of 0.05 µg/L, using LC/MS/MS for quantification and confirmation.

BAS 650 F



Principles of the Method and Validation:

BASF method no. 574/0, as obtained by the sponsor, using LC/MS/MS was employed.

BAS 650 F is extracted from a 100 g water sample aliquot with dichloromethane. An aliquot is reduced to dryness and dissolved in acetonitrile/water (50/50, v/v). The final determination is performed using HPLC/MS/MS, resulting in a limit of quantification (LOQ) of 0.05 µg/kg.

For the analyte two parent-daughter ion transitions were monitored by LC/MS/MS for quantification and quantitative confirmation.

The method achieves a limit of quantification (LOQ) of 0.05 µg/kg.

Method validation was accomplished by analyzing for surface water and for tap (drinking) water 2 blank control specimens, 5 replicate specimens fortified at LOQ, and 5 replicate specimens fortified at 10xLOQ.

2. EXPERIMENTAL

Materials and Methods

The materials, chemicals and the equipment used during this study were of similar specifications as described in the related technical procedure (TP) method no. 574/0 (see Appendix 2) in Section 3. Acetic acid was used as a modifier for both HPLC eluents instead of formic acid as described in the TP.

For characterization of water samples the following equipment was used: Total hardness test (Merck, Germany) and pH meter (Denver Instr. Comp., USA).

Test and Reference Items

The analytical standard used was obtained by the Sponsor. See Appendix 1 for information provided.

Test System

Drinking (tap) water drawn at PTRL. The water was clear, had no smell, pH was 7.6, total water hardness was 2.2 mmol/L corresponding to 12.3 °dH.

Surface (river) water was collected on 08-Apr-08 from a pond in Bad Schussenried, located in Southern Germany. The appearance of the water was yellowish. The water was characterized for physical and chemical properties as follows: pH 7.2, total water hardness: 14.2°d (Deutsche Härtegrade, 2.5 mmol/L), total organic carbon (TOC): 11 mg/L, dissolved organic carbon (DOC): 3.7 mg/L, turbidity: 105 NTU, silt content: 430 mg/L.

Analytical Procedure

The analytical procedure of method no. 574/0 is described in Section 4 of the technical procedure method no. 574/0 (see Appendix 2).

LC/MS/MS Analysis

The following HPLC/MS/MS instrumentation, HPLC method and MS/MS conditions were used:

HPLC System	Agilent 1100 HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.
HPLC Column	Waters XTerra C_{18}, 50 mm length, 4.6 mm i.d., 3.5 µm particle size. Pre-column: Phenomenex C_{18} RP, 4 x 3 mm. Column oven 30 °C.
Injection Volume	50 µL.
HPLC Method	Solvent A: 0.1 % acetic acid in water Solvent B: 0.1 % acetic acid in acetonitrile Mobile Phase Composition: Time (min) / Flow rate (µL/min) / % A / % B 0.00 / 400 / 90 / 10 7.00 / 400 / 0 / 100 9.00 / 400 / 0 / 100 9.10 / 400 / 90 / 10 12.0 / 400 / 90 / 10
MS System	Applied Biosystems MDS Sciex API 3000 triple quadrupole LC/MS/MS system with TurboIonspray (ESI) source; Analyst Software 1.4.2
Electrospray Ion Source Conditions	Polarity: Positive Source temperature: 450°C Curtain gas (CUR): 12 Nebulizer gas (NEB): 14 Ion spray voltage (IS): 4500 V Collision gas (CAD): 4 Entrance potential (EP): 10 V Resolution Q1 and Q3: Unit Dwell times: 500 msec
MS/MS Conditions	MRMs proposed for quantification (Q) respectively confirmation (C): Analyte: BAS 650 F Q1 Mass (amu) / Q3 Mass (amu) / DP (V) / FP (V) / CE (eV) / CXP (V) 276.3 / 149.1 (Q) / 80 / 300 / 50 / 13 276.3 / 176.2 (C) / 80 / 300 / 44 / 15

External calibration was used for quantification and confirmation of the analyte by LC/MS/MS. Calibrations were established with standard solutions prepared in acetonitrile /

water (1/1, v/v) injected interspersed with water extracts. The calibrations ranged from 0.01 ng/mL to 5 ng/mL with 6 concentration levels.

Linear regression equations were generated with 1/x weighting, resulting in calibration functions with excellent correlation ($r \geq 0.999$), as exemplified in Figure 1 with calibration functions and diagrams.

LC/MS/MS chromatograms of standard solutions are shown in Figure 2.

Figure 3 (tap water) and Figure 4 (surface water) give examples of LC/MS/MS chromatograms of fortified (10xLOQ: 0.50 µg/kg, LOQ: 0.05 µg/kg) and blank control water specimens.

Calculation of Residue and Example

Calculations were performed by Excel with full precision; discrepancies may arise when recalculated with pocket calculator.

For the calculation of residues the following formula was used:

$R = c_{End} \times (V_{Ex} \times V_{End} / V_1 \times W)$

$= c_{End} \times$ Multiplier M

Where:

R: Analyte residue in µg/kg.

c_{End}: Final concentration of analyte in extract in ng/mL.
(where multiple injections were evaluated: mean).

W: Sample weight: 100 g.

V_{Ex}: Volume of extraction solvent: 25 mL.

V_1: Aliquot of V_{Ex}: 10 mL.

V_{End}: Volume of final extract used for LC/MS/MS: 20 mL.

M: Multiplier: 0.50.

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$Rec. = (R / R_{fortified}) \times 100$ %.

The calculation is exemplified with a tap water specimen fortified at 0.05 µg/kg or LOQ (P1519-17, see Table 1) with BAS 650 F.

The 100 g (W) water specimen was extracted for 30 min with 25 mL of dichloromethane (V_{Ex}) on a shaker. After separation of liquids an aliquot (V_1 = 10 mL) of the dichloromethane phase was taken, concentrated to dryness and re-dissolved with 20 mL (V_{End}) of acetonitrile/water (1/1, v/v). An aliquot thereof was used for LC/MS/MS determination.

The final extract was examined for BAS 650 F by LC/MS/MS in run file P1519api#012 (Figure 3, middle). The Analyst software used a calibration function which was established by injecting calibration solutions interspersed with final extracts to calculate a final BAS 650 F

concentration c_{End} of 0.095 ng/mL (276 m/z → 149 m/z), respectively, 0.097 ng/mL (276 m/z → 176 m/z). Thus:

R = c_{End} x (V_{Ex} x V_{End}) / (V_1 x W)
= c_{End} x Multiplier M
= 0.095 ng/mL x (25 mL x 20 mL) / (10 mL x 100 g)
= 0.095 ng/mL x 0.5
= 0.0475 ng/g or µg/kg

The result gave a recovery of 95 % for the ion transition 276 m/z → 149 m/z used for quantification.

Stability of residues in water samples

The storage stability of residues of BAS 650 F in water samples will be investigated in a separate study which is not started up to now. Preliminary details of this study are given in Reference 1.

1 INTRODUCTION

1.1 Scope of the method

This method is used to determine the residues of Reg.no. 4993353 in water samples. The analyte is a new fungicide against Phytophthora in tomatoes and Plasmopara in grapes. It considers the progress of development in sample preparation and HPLC/MS.

1.2 Principle of the method

A 100 g water sample aliquot is fortified with 10 g NaCl. After dissolution of the salt, 25 ml of dichloromethane are added. The sample is shaken with 200 rpm for 30 minutes on a mechanical shaker. After separation of the liquid phased, a 10 ml aliquot of the organic phase is taken and evaporated to dryness. The residue is dissolved in acetonitrile + water 50 + 50. An aliquot is injected into a HPLC/MS system for final determination. The limit of quantification of the method is 0.05 µg/kg.

1.3 Specificity

The method allows the specific determination of Reg.no. 4993353 in water samples.

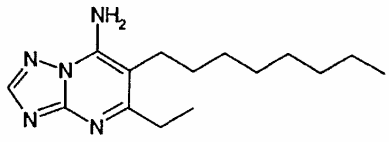
1.4 Safety

1. Due to the limited information about human toxicologic effects, Reg.no. 4993353 should be handled with care and responsibility.
2. Acetonitrile is flammable and should not be used near heat, sparks or open flames. Dichloromethane and acetonitrile are toxic. Formic acid is corrosive.
3. All solvents should be used only in well ventillated laboratories or cabinets.
4. Protective glasses and clothing should be worn during all laboratory procedures.
5. Disposal of samples and standards must be done in compliance with on-site safety policies and procedures.

2 TEST AND REFERENCE ITEMS

2.1 Reg.no. 4993353

BAS Code:	PA-Pre
Chemical name	5-ethyl-6-octyl[1,2,4]triazolo[1,5-a]pyrimidin-7-amine
Reg. no.:	4993353
Structural formula:	



Chemical formula:	$C_{15}H_{25}N_5$
Molucular weight:	275.4 g/mol
Purity:	100 %
Lot.No.:	NL 6, supplied by BASF, APR/HP, Li 721
Stability:	stability is not yet known, but compound is expectet to be stable for 2 years from date of analysis (Expiration date 03/2007)
Purpose:	used for fortification and calibration experiments

2.2 Storage stability of residues of test item in water samples

The stability of Reg.no. 4993353 residues in tap water in the dark at about 4°C was investigated. During the method development period, the substance was dissolved in tap water and the samples were analysed after different periods of storage. The longest storage interval was 16 days. Up to this time, no significant decrease was observed.

3 MATERIALS AND METHODS

Note: The materials, chemicals and the equipment specified below were used during method development. They are specified as examples only and may be substituted with supplies of similar specifications. If the use of supplies other than those stated is intended, applicability to this method must be confirmed prior to method validation and/or routine analysis.

3.1 Equipment for extraction and sample clean-up

Equipment	Size, Description	Manufacturer, Supplier	Order no.
Glass bottles screw cup	250 ml	e.g. BASF	4363462, 4363494
Volumetric flasks	25, 20, 10, 5 ml		
Bulb pipettes	0.5, 1, 2, 2.5 ml		
Graduated cylinder	100 ml, 25 ml		
Tapered flask	50 ml		
Volumetric pipettes	5, 10, 25 ml		
Balance	LP5200P	Sartorius AG, Germany	
Analytical balance	AT261 Delta Range	Mettler, Germany	
Vacuum rotary evaporator with vacuum pump controller and water bath	LABOROTA 4003	Heidolph, Germany	
Vacuum pump	MZ2C	Vacuubrand, Germany	
Mechanical shaker	SM 25	Bühler, Tübingen	
Ultrasonic water bath	Transsonic 820/H	Labotec, Germany	
HPLC vials	250 µL		
HPLC/MS	System specified in chapter 4.4	Agilent, USA	

3.2 Reagents

3.2.1 Chemicals

Note: All chemicals used must be at least of "analytical grade" or must meet equivalent specifications.

Chemical	Grade	Manufacturer/Supplier	Order no.
Water (ddH_2O)*)	Millipore grade, taken from the in-house water pipe		
Dichloromethane (DCM)	Suprasolv	Merck, Germany	1.06054.2500
Sodiumchloride (NaCl)	Pro analysi	Merck, Germany	
Methanol (MeOH)	LiChrosolv	Merck, Germany	1.06018.2500
Acetonitrile (ACN)	Lichrosolv for LC	Merck, Germany	1.00030.2500
Formic acid (HCOOH)	pro analysi 98 % - 100 %	Merck, Germany	1.00264

*) double-deionised water

3.2.2 Solutions and solvent mixtures

Description	Composition
HPLC eluent A	Water/HCOOH 1000/1 (v/v)
HPLC eluent B	ACN/HCOOH 1000/1 (v/v)
Final volume solution	Water/acetonitrile 50/50 (v/v)

3.2.3 Fortification solutions

Note: The concentrations given below are proposals. Different concentrations can be used depending on the expected residue range.

Compound	Stock solution concentration	Dilutions with final volume solution to concentrations of
Reg.no. 4993353	500 µg/ml in MeOH	5 ng /ml, 50 ng/ml

3.2.4 Calibration solutions

Note: The concentrations given below are proposals. Different concentrations can be used depending on the expected residue range.

Compound	Stock solution concentration	Dilutions with final volume solution to concentrations of
Reg.no. 4993353	500 µg/ml in MeOH	5 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml, 250 pg/ml, 500 pg/ml

4 ANALYTICAL PROCEDURE

4.1 Sample preparation and fortification

Weigh about 100 g of water into glass bottle (pre weigh bottle on balance as base of exact sample weight).

In the case of fortification samples, fortify untreated water sample with 1 ml of the spiking solution with analyte concentrations of 0.005 µg/ml (for residue at limit of quantification) or higher.

The correlation between the concentration of the spiking solution and the resulting final analyte concentration in the sample is shown below:

Sample weight	Concentration of spiking solution	Volume of spiking solution	Level of fortification
100 g	-	-	0.00 µg/kg (blank)
100 g	0.005 µg/ml *)	1 ml	0.05 µg/kg

*) The fortification level 0.05 µg/kg is the proposed limit of quantification.

4.2 Analyte extraction

Add 10 g of Sodiumchloride with aid of a funnel to the water sample.

Mix well in order to solve the solids.

Add 25 ml of dichloromethane with a bulb pipette.

Extract liquids for 30 minutes at 225 rpm on a mechanical shaker.

Allow the phases to separate. In the case of separation problems, sonicate or centrifuge the liquid phases.

Take aliquot of 10 ml from the dichloromethane phase by a bulb pipette through the aqueous phase and transfer it into a 50 ml round bottom flask.

4.3 Preparation of the injection volume for HPLC/MS

Concentrate the extract of chapter 4.2 right to dryness at a water bath temperature of about 40 °C by vacuum rotary evaporation.

Add an appropriate volume of final volume solution depending on the residue level expected. For residues expected at the limit of quantification, a volume of 20 ml is recommended (depending on the instrument performance). For higher residues, dilutions can be prepared in advance which should be analysed before the more concentrated equivalents.

Dissolve residue thoroughly with aid of sonication.

Transfer an aliquot of this solution into a 250 µl HPLC vial.

Inject 50 µl of this solution into a HPLC/MS system.

Note:
It is advisable to verify the retention times and the sensitivity of the analyte on the chromatograpy systems prior to each analytical series. For this, appropriate standard solutions can be injected into the chromatography system to verify the peak retention time, the resolution and the sensitivity of the reference substance and show the stability of the system. The retention time depends strongly on the type and the dimensions of the chromatography system.

The equipment listed below was used for the test of the method in water. It may be substituted however by equipment with similar specifications.

4.4 HPLC/MS instrumentation and conditions

Instrument	HPLC pump	Agilent, Series 1100, Binary Pump	
	Autosampler	Agilent, Series 1100	
	System software	Agilent Chemstation	
	HPLC-MSD	Agilent, Series 1100	
Analytical column	Packing material	Luna C8, Mercury MS, Phenomenex	
	Length	20 mm	
	Inner diameter	4.0 mm	
	Particle size	3 µm	
HPLC conditions	Flow rate	800 µl/min	
	Injection volume	50 µl	
Gradient	Time	Eluent A [%]	Eluent B [%]
	0 min	100	0
	6 min	0	100
	8 min	0	100
	8.1 min	100	0
	10 min	100	0
Mass	SIM (pos.)	276.3	
Retention time	Reg.no. 4993353	About 4 min	

Examples of HPLC/MS chromatograms are given throughout Figure 1 to Figure 5.

4.5 Quantification of the results

If the sensitivity and stability of the chromatography system and the analyte retention times are checked, calibration standard solutions and sample can be measured alternately. For all quantification purposes, peak areas are taken into account.

A linear regression curve can be calculated from the calibration standard signals.

4.6 Limit of detection and limit of quantification

The lowest standard concentration is supposed to be the limit of detection. This means, for a concentration of 5 pg/ml, the signal/noise ratio should be equal or better than 3/1. The limit of detection depends on the instrumentation and on the conditions used, but during method development, these conditions were met. The limit of detection is set to be 2.5 ng/kg, based on the lowest calibration standard signal.

The limit of quantification of the method is 0.05 µg/kg.

5 CALCULATION OF THE RESULTS

5.1 Principle

The evaluation makes use of calibration curves recorded during each analytical run. Area signals of Reg.no. 4993353 are plotted against the injected ng amont of the corresponding calibration standard. Using this curve, injected amounts can be calculated from signals of unknown samples. The injected amounts are needed for the residue calculations as outlined below. A calculation example is given in Appendix 1.

5.2 Calculation of residues

The residues of Reg.no. 4993353 in µg/kg are calculated with the following formula:

$$R = \frac{V_{end} \times C_B}{V_i \times S_M}$$

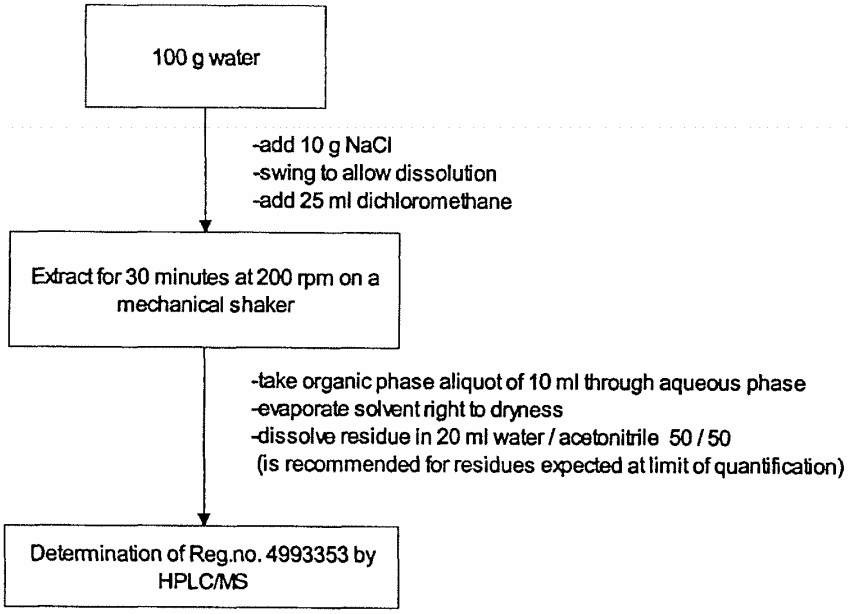
R	=	Residue of Reg.no. 4993353 in the water sample [µg/kg]
V_{end}	=	Final volume of the extract prepared for HPLC/MS injection [ml]
C_B	=	Amount of Reg.no. 4993353 in the injection volume as read from the calibration curve [ng]; in cases, the analyte signal is 0, the amount injected is 0, too.
V_i	=	Extract volume injected into HPLC/MS (aliquot of V_{end}) [µl]
S_M	=	Weight of the water sample extracted [kg]

5.3 Calculation of recoveries

The recoveries of samples spiked with Reg.no. 4993353 are calculated with the following formula:

$$\text{Recovery [\%]} = \frac{(R \text{ in fortified sample} - R \text{ in control sample})}{\text{Amount fortified [ug/kg]}} \times 100$$

6 FLOW DIAGRAM OF METHOD 574/0



7 TIME REQUIREMENT FOR ANALYSIS

Beginning with addition of NaCl and ending with the preparation of the final volume for injection, a sample set containing 12 samples, can be prepared for HPLC/MS injection within 5 hours. HPLC/MS analysis and times for calculating the results has to be taken additionally into account.

8 POTENTIAL PROBLEMS

Liquid phase partition
In case of emulsion formation, the phases may separate by applied sonication. If this measurement don´t solve the problem, the phases can be separated by centrifugation (not necessary during method development).

10 CONFIRMATORY TECHNIQUES

As a confirmatory technique, the determination can be conducted by /MS/MS detection. As an example, the following instrument parameters can be used:

Instrument	HPLC pump	Agilent, Series 1100, Binary Pump	
	Autosampler	Perkin Elmer PE 200	
	System software	Analyst 1.4.1	
	HPLC-MS/MS	API 3000, PE Sciex Instruments	
Analytical column	Packing material	Betasil C18, Thermo Electron	
	Length	100 mm	
	Inner diameter	2.1 mm	
	Particle size	5 µm	
HPLC conditions	Flow rate	400 µl/min	
	Injection volume	50 µl	
Gradient *	Time	Eluent A [%] **	Eluent B [%] **
	0 min	60	40
	3 min	0	100
	6 min	0	100
	6.1 min	60	40
	10 min	60	40
MS conditions ***	Ionization	Turbo Spray (MRM positive)	
	Prochloraz	Masses: 276 (Q1), 176 (Q3) or 276 (Q1), 190 (Q3)	

* During analysis, a waste switching technique was installed, insuring that the flow from the analytical column was passed into the MS/MS detector between retention time 3.2 and 5.3 minutes (elution of the analytes after about 4 min). Before and after this period, the flow was passed into waste. To ensure a constant flow into the MS, a second pump (Agilent 1100 LC quaternary pump) delivered a constant flow of a solvent (300 µl/min) and a valve controlled the flows into waste respectively MS.

** HPLC eluent A: Water / HCOOH 1000 / 1 (v/v)
HPLC eluent B: methanol / HCOOH 1000/1 (v/v)

*** evaluation masses are underlined