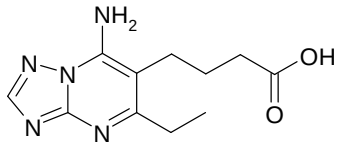
1. INTRODUCTION

Objective:

The objective of this study was to validate an analytical method for the determination of BAS 650 F metabolites M650F01, M650F02, M650F03, and M650F04 in water at a limit of quantification (LOQ) of 0.05 µg/kg (per analyte), using LC/MS/MS for quantitation and confirmation..

M650F01



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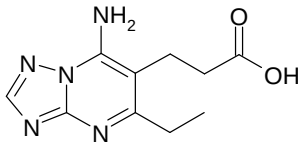

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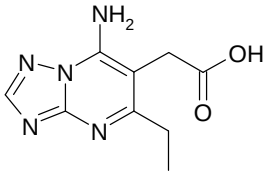
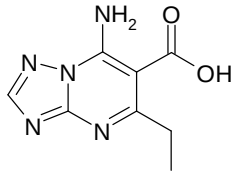


M650F04



Principles of the Method and Validation:

BASF method L0113 using LC/MS/MS was employed. The method is described in the related technical procedure (see Appendix 2).

The analytes are extracted from water by solid-phase extraction using Strata-X-C cartridges to retain all analytes from a 10-g aliquot of water, with subsequent elution by 10 mL of acetonitrile/NH_3 25 % (90/10, v/v). The eluate is concentrated to dryness and re-dissolved in 2 mL of acetonitrile /water (10/90, v/v). An aliquot is used for the final determination of the BAS650F metabolites M650F01, M650F02, M650F03 and M650F04 by LC/MS/MS.
For all four analytes 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 drinking (tap) 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 are described in Section 2 of the technical procedure of Method L0113 (see Appendix 2). They are specified as examples only and may be substituted with supplies of similar specifications.
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 test and reference items used are described in Section 1.4 of the technical procedure of Method L0113 (see Appendix 2). The certificates of analysis of the analytes used in this study are shown in Appendix 1 and were obtained by the sponsor.

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 L0113 is described in Section 3 of the technical procedure Method L0113 (see Appendix 2).

Calculation of Residues: 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:

$R = c_{End} \times (V_{Ex} \times V_{End}) / (V_1 \times W) / 1000$ ng/µg

$= c_{End} \times$ 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: Water sample weight: 10 g.

V_{Ex}: Volume of extraction solvent: 10 mL.

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

V_{End}: Volume of final extract used for LC/MS/MS: 2.0 mL.

M: Multiplier: 0.20.

The calculation is exemplified with a surface water specimen fortified at 0.05 µg/kg or LOQ (P1483-37, see Table 2) with M650F01 (and the three other BAS650F metabolites).

The 10 g (W) specimen aliquot was enriched on an Strata-X-C SPE cartridge, the analytes eluted by 10 mL (V_{Ex}) of acetonitrile/NH_3,(90/10, v/v), which was quantitatively transferred (V_1= 10 mL) and reduced to dryness. Residues were re-dissolved in a final volume of 2 mL acetonitrile/water (V_{End}, 10/90, v/v), an aliquot thereof was used for LC/MS/MS determination.

The final extract was examined for M650F01 by LC/MS/MS in run file P1483API#064 (Figure 13, middle). The Analyst software used a calibration function which was established by injecting calibration solutions interspersed with final extracts to calculate a final M650F01 concentration c_{End} of 0.206 ng/mL (250 m/z → 232 m/z), respectively, 0.228 ng/mL (250 m/z → 149 m/z). Thus:

R = c_{End} x (V_{Ex} x V_{End}) / (V_1 x W) / 1000 ng/µg

= c_{End} x Multiplier M

= 0.206 ng/mL x (10 mL x 2 mL) / (10 mL x 10 g) / 1000 ng/µg

= 0.206 ng/mL x 0.2

= 0.0412 µg/kg

The result gave a recovery of 82 % for the ion transition 250 m/z → 232 m/z used for quantification.

Stability of residues in water samples

The storage stability of residues of all analytes 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 residues of BAS 650 F metabolites M650F01, M650F02, M650F03 and M650F04 in water samples.

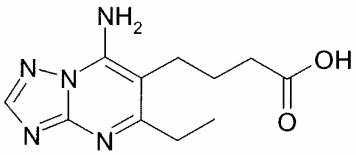
1.2 Principle of the method

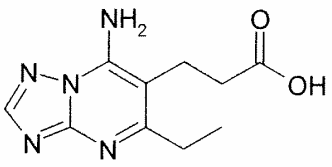
The analytes are extracted from water by solid-phase extraction using Strata-X-C cartridges to retain all analytes from a 10-g aliquot of water, with subsequent elution by 10 mL of acetonitrile/NH_3 (90/10, v/v). The eluate is concentrated to dryness and re-dissolved in 2 mL of acetonitrile /water (10/90, v/v). An aliquot is used for the final determination of the BAS650F metabolites M650F01, M650F02, M650F03 and M650F04 by LC/MS/MS. The limit of quantification of the method is 0.05 µg/kg.

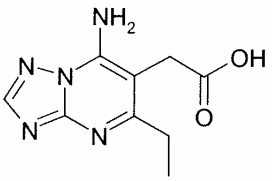
1.3 Specificity

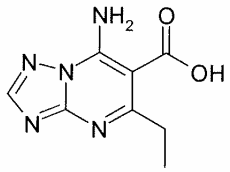
The method allows the specific determination of BAS 650 F metabolites M650F01, M650F02, M650F03 and M650F04 in water samples.

1.4 Test and Reference Items

Reg. No	Internal (Metabolite) Code	Chemical formula	Molecular mass
5178872	M650F01	$C_{11}H_{15}N_5O_2$	249.3 g/mol
Chemical Name (IUPAC)	4-(7-amino-5-ethyl[1,2,4]triazolo[1,5-a]pyrimidin-6-yl) butanoic acid		
Structural formula			

Reg. No	Internal (Metabolite) Code	Chemical formula	Molecular mass
5178871	M650F02	$C_{10}H_{13}N_5O_2$	235.3 g/mol
Chemical Name (IUPAC)	3-(7-amino-5-ethyl[1,2,4]triazolo[1,5-a]pyrimidin-6-yl) propanoic acid		
Structural formula			

Reg. No	Internal (Metabolite) Code	Chemical formula	Molecular mass
5178870	M650F03	$C_9H_{11}N_5O_2$	221.2 g/mol
Chemical Name (IUPAC)	(7-amino-5-ethyl[1,2,4]triazolo[1,5-a]pyrimidin-6-yl)acetic acid		
Structural formula			

Reg. No	Internal (Metabolite) Code	Chemical formula	Molecular mass
5211623	M650F04	$C_8H_9N_5O_2$	207.2 g/mol
Chemical Name (IUPAC)	7-amino-5-ethyl[1,2,4]triazolo[1,5-a]pyrimidin-6-carboxylic acid		
Structural formula			

2 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.

2.1 Equipment for sample extraction

Equipment	Size, Description	Manufacturer, Supplier
Glass bottles	20 ml	Amchro, Germany
pH sticks	pH fix 4.5 - 10	Fisher Brand, Germany
Volumetric flasks	Various sizes	Various manufacturers
Volumetric pipettes	Various sizes	Various manufacturers
Analytical balance	RC 210 D	Sartorius AG, Germany
SPE station	Baker SPE	Macherey&Nagel, Germany
Evaporation flasks	Various sizes	Various manufacturers
Vacuum rotary evaporator with vacuum pump controller and water bath	Rotavapor 114	Büchi, Switzerland
Ultrasonic water bath	Transsonic 700	Schmidbauer, Germany
HPLC vials	1800 µL	
HPLC/MS/MS	System specified in chapter 3.4	Agilent, USA

2.2 Reagents

2.2.1 Chemicals

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

Chemical	Grade / Purity	Manufacturer/Supplier
Water (ddH_2O)*)	Millipore grade	Ptrl Europe GmbH
Methanol (MeOH)	HPLC grade	Promochem, Germany
Acetonitrile (ACN)	HPLC grade	Promochem, Germany
Ammonia solution (NH_3)	25 %	Merck, Germany
Acetic acid (CH_3COOH)	100 %	Merck, Germany

*) double-deionised water

2.2.2 Solutions and solvent mixtures

Description	Composition
HPLC eluent A	Water/CH_3COOH 1000/1 (v/v)
HPLC eluent B	ACN/ CH_3COOH 1000/1 (v/v)
Final volume solution	Water/acetonitrile 90/10 (v/v)

2.2.3 Stock Solutions

Stock solutions were prepared by accurately weighing the test/reference items to the nearest 0.1 mg into individual 10-mL volumetric flask and then dissolving the analytes in methanol to obtain for each analyte a concentration of 1.0 mg/mL, as exemplified below:

Compound Name	Purity	Weight	Sol. Vol.	Conc.
	%	mg	mL	mg/mL
M650F01	79.9	12.5	10	1.0
M650F02	80.1	12.5	10	1.0
M650F03	83.5	12.0	10	1.0
M650F04	95.8	10.4	10	1.0

2.2.4 Fortification solutions

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

Compounds	Stock solution concentration	Take equal aliquots of each individual stock solution and prepare dilutions with methanol to concentrations of:
M650F01, M650F02, M650F03, M650F04	1000 µg/ml in MeOH	5 ng/mL and 50 ng/mL

2.2.5 Calibration solutions

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

Compounds	Stock solution concentration	Take equal aliquots of each individual stock solution and prepare dilutions with final volume solution to concentrations of:
M650F01, M650F02, M650F03, M650F04	1000 µg/ml in MeOH	50 pg/ml, 100 pg/ml, 500 pg/ml, 1 ng/ml, 5 ng/mL

3 ANALYTICAL PROCEDURE

3.1 Sample preparation and fortification

10 g (or 10 mL) aliquots of water were weighted (or measured) (pH should be about 7, adjust with HCl or NH_3, if necessary).

In the case of fortification samples, fortify untreated water sample with 0.10 ml of the spiking solution with analyte concentrations of 5 ng/ml (for residue at limit of quantification: 0.05 µg/kg)) or with analyte concentrations of 50 ng/ml for ten times the limit of quantification (0.5 µg/kg).

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

Sample volume	Concentration of spiking solution	Volume of spiking solution	Level of fortification
10 g	-	-	0.00 µg/kg (blank)
10 g	5 ng/ml *)	0.10 ml	0.05 µg/kg
10 g	50 ng/ml	0.10 ml	0.5 µg/kg

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

3.2 Extraction of analytes

Strata-X-C SPE cartridges are placed on SPE station(s) equipped with Teflon stop cocks, vacuum manifold(s) attached to water suction pump(s) or vacuum pumps.

SPE cartridges are pre-conditioned with 6 mL of acetonitrile and 6 mL of Millipore water.

The 10-g water specimens (adjust pH to about 7 with HCl or NH_3, if necessary) are added onto the cartridge and the flow is adjusted to approximately 1 – 2 mL/min. Once all water is drawn through, the cartridge is dried by applying vacuum. The stop cocks are closed and the extracted water discarded.

10 mL of acetonitrile/NH_3 (25 %) (90/10, v/v) are added and drawn through the cartridges at flows of 1 – 2 mL/min to elute all four analytes from the Strata-X-C material.

The 10-mL eluate is collected and transferred in pear shaped evaporation flasks.

3.3 Preparation of the final extract for HPLC/MS/MS

The eluate of chapter 3.2 is reduced to a small amount of water by rotary evaporation (water bath temperature about 50°C), which is then further reduced to dryness using a gentle stream of nitrogen.

The residue is re-dissolved thoroughly with the aid of sonication in 2 mL of acetonitrile/water (10/90, v/v): Add first 200 µL acetonitrile, sonicate and then add 1800 µL water with subsequent sonication.

An aliquot is transferred into a 1.8 mL HPLC vial.

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

3.4 HPLC/MS/MS instrumentation and conditions

Instrument	Mass spectrometer	PE Sciex API 4000				
	HPLC pump	Agilent, Series 1200, Binary Pump				
	Autosampler	HTC PAL				
	System software	Analyst Version 1.4.2				
Analytical column	Packing material	XTerra C_{18}, Waters				
	Length	50 mm				
	Inner diameter	4.6 mm				
	Particle size	3.5 µm				
	Column Oven Temperature	30°C				
HPLC conditions	Flow rate	500 µl/min				
	Injection volume	50 µl				
Gradient Pre-equilibration time of 2 min with 500 µL/min at 90% A : 10% B.	Time	Eluent A [%]	Eluent B [%]			
	0 min	90	10			
	7 min	0	100			
	9 min	0	100			
	9.1 min	90	10			
	11 min	90	10			
Ion Source/Polarity	Turbo Ion Spray (ESI), positive Ionization					
Electrospray Ion Source Conditions	Source temperature: 550°C Curtain gas (CUR): 20 Nebulizer gas (GS1): 40 Turbo gas (GS2): 70 Ion spray voltage (IS): 4500 V Collision gas (CAD): 5 Entrance potential (EP): 10 V Resolution Q1 and Q3: Unit Dwell times: 100 msec (M650F01, M650 F02) 200 msec (M650F03, M650F04)					
MS/MS Conditions	Analyte	Q1 Mass	Q3 Mass	DP (V)	CE (V)	CXP (V)
	M650F01	250.2	232.0	46	31	22
			149.1	46	49	12
	M650F02	236.0	176.1	60	36	16
			218.2	60	28	15
	M650F03	222.2	176.1	41	31	16
			204.1	61	23	16
	M650F04	208.2	190.2	37	24	15
			123.0	37	35	11
Expected Retention Times	M650F01	3.6 min				
	M650F02	2.5 min				
	M650F03	2 min				
	M650F04	2.9 min				

Note:

Instruments with similar specifications may substitute the equipment listed above. If the use of material with specifications other than those stated is intended, applicability of the new equipment for this method must by confirmed

The instrument conditions, injection volume, column and gradient steps may be modified, but any changes must be recorded in the raw data.

Dwell-Time depends on the number of transitions.

3.5 Quantification of the results

If the sensitivity and stability of the chromatographic system and the analyte retention times are checked, calibration standard solutions and samples 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.

3.6 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 0.05 ng/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 0.01 µg/kg, based on the lowest calibration standard signal.

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

4 CALCULATION OF RESULTS

4.1 Principle

The evaluation makes use of calibration curves recorded during each analytical run. Area signals of the analytes 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.

4.2 Calculation

The individual concentrations of each analyte in the sample material are calculated as shown below:

$R = C_{End} \times (V_{Ex} \times V_{End})/(V_1 \times W \times 1000\ ng/\mu g)$

$= C_{End} \times$ Multiplier M

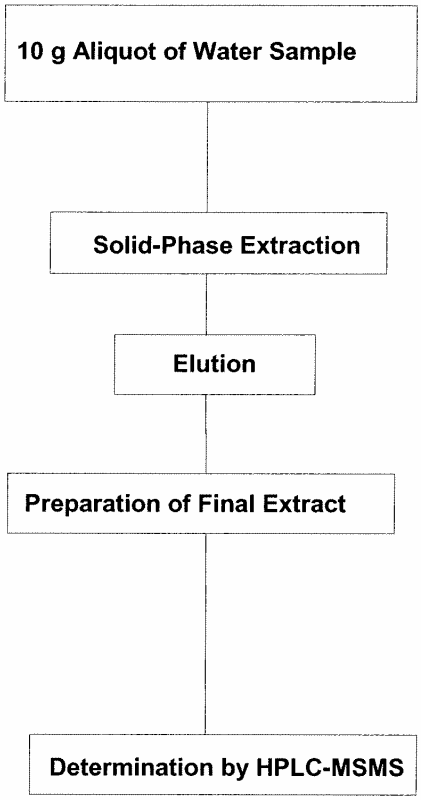
Where:

R: Analyte residue in µg/kg or ppb.
C_{End}: Final concentrations of analyte in extracts, in ng/mL.
(where multiple injections were evaluated: average).
W: Weight of the water sample extracted by SPE: 10 g
V_{Ex}: Volume of ACN/NH_3 25% (9/1, v/v) SPE eluate: 10 mL
V_1: Volume of SPE eluate reduced to dryness: 10 mL
V_{End}: Final volume: 2.0 mL
1000: Divisor used to adjust for dimensions, converts ng to µg.
Multiplier M: 0.2 (for residues expected to be at the LOQ)

Recoveries (in %) are calculated as follows:

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

5 FLOWCHART OF METHOD L0113



-the pH value should be approx. 7 (adjust optionally using HCl or NH_3)

-pre-condition the SPE cartridges (Phenomenex Strata-X-C, 200 mg adsorbens, 6 mL volume) using 2x3mL acetonitrile and 2x3 mL Millipore water

-add the 10-mL aliquot of water onto the cartridge (flow ~ 1-2 mL/min) and dry the cartridge under vakuum

-elute the cartridge with 10 mL (2x5 mL) acetonitrile/NH_3 25 % (90/10, v/v)

-reduction of volume: the eluate is reduced to dryness
-re-dissolve using 2 mL of acetonitrile/water (1/9, v/v)

6 Time Management and Time Requirement for Analysis

Beginning with the pre-conditioning of SPE cartridges 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 8 hours. HPLC/MS/MS analysis (approximately 13 minutes per LC-MS/MS run) and evaluation of results has to be taken additionally into account.

7 POTENTIAL PROBLEMS

No problems during the analytical procedure described herein were observed.

8 CONFIRMATORY TECHNIQUES

The LC/MS/MS determination for the BAS 650 F metabolites M650F01, M650F02, M650F03 and M650F04, monitoring at least two parent-daughter ion transitions, is a highly selective detection technique. Therefore no additional confirmation is required.