MRID: 50105408

STUDY TITLE

Method Development and Validation of an Analytical Method for the Determination of BAS 720 H and its 2 Metabolites Reg. No 4110603 and Reg. No 4110542 in Water (Analytical Method L0209)

EPA Guideline(s)

OPPTS 850.6100

Study Identification Number(s)

BASF Study ID: 391110
SGS Project No.: IF-13/02484111

BASF Registration Document Number

2015/7008009

3 Information about the Study

3.1 Test System

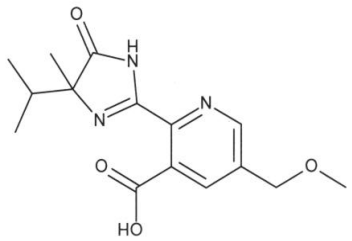
The specimen of surface water was obtained from a natural surface water course known as Burbach located in Dietzhoelztal, D-35716 in Germany. The specimen of ground water was obtained from a well located in Glashuetten, D-61479 in Germany . The specimens were given the following numbers:

ground water:	130206571
surface water:	130160769

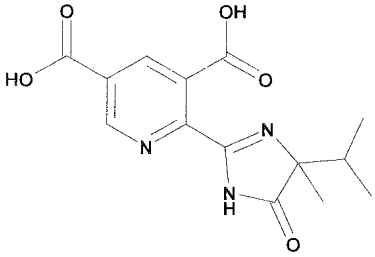
See also Table 1 and 3.

4 Test / Reference Items

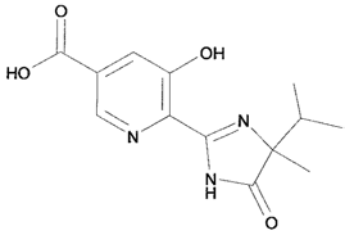
4.1 BAS 720 H

Common name	Imazamox
Reg. No.	4096483
IUPAC-Name	(RS)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid
CAS-No.	114311-32-9
Molecular formula	$C_{15}H_{19}N_3O_4$
Structure	
Molecular weight	305.3
Lot No.	AC12820-7
Appearance	solid white (assayed by SGS-IF)
Purity	99.5 %
Storage advice	keep in refrigerator or freezer
Expiry Date	01 May 2021

4.2 Reg. No. 4110542

CL-No.	312622
Reg. No.	4110542
IUPAC-Name	2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)pyridine-3,5-dicarboxylic acid
CAS-No.	146953-32-4
Molecular formula	$C_{14}H_{15}N_3O_5$
Structure	
Molecular weight	305.3
Lot No.	L82-7
Appearance	solid white (assayed by SGS-IF)
Purity	88.4 %
Storage advice	keep in refrigerator or freezer, hygroscopic (keep away from humidity)
Expiry Date	November 01, 2013

4.3 Reg. No. 4110603

CL-No.	354825
Reg. No.	4110603
IUPAC-Name	5-hydroxy-6-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid
CAS-No.	-
Molecular formula	$C_{13}H_{15}N_3O_4$
Structure	
Molecular weight	277.3
Lot No.	L67-144
Appearance	solid yellow-green (assayed by SGS-IF)
Purity	98.2 %
Storage advice	Keep at room temperature (typically + 25°C) or cooler
Expiry Date	July 01, 2020

These reference items and the respective data were supplied by the sponsor.
Stock solutions and working solutions of the reference items were stored between + 2 °C and + 8 °C in capped vials protected from light.

5 Purpose of the Study

The scope of this study was to validate an analytical method for the determination of BAS 720 H, Reg. No. 4110603 (CL-No. 354825) and Reg. No. 4110542 (CL-No. 312622) in water according to the guidelines mentioned in chapter 1.1 at a limit of quantification of 0.025 µg/L using LC-MS/MS for quantitation and confirmation.

6 Deviation to Study Plan

The analytical method L0209 was validated for determination of BAS 720 H and its metabolites Reg. No. 4110542 and 4110603 in water instead of the BASF methods M3515 (BASF Doc ID 2002/7006086) and BASF Method D1102.

7 Analytical Phase

7.1 Analytical Method

The used method L0209 was based on the existing BASF methods M 3513, BASF Doc ID 2002/7006086, *"LC/MS Determination and LC/MS/MS Confirmatory Method for Residues of CL 299263, CL 312622 and CL 354825 in surface water*" and BASF Method D1102; *"Method for Determination of BAS 720 H and its Metabolites CL 312622 and CL 354825 Residues in Soil Using LC-MS/MS"*. This was done for the validation of an Analytical Method for the Determination of BAS 720 H and 2 Metabolites in Water.

Principle of the Method
The test systems ground water and surface water will be prepared for isolation of residues by solid phase extraction (SPE). The estimated limit of quantitation (LOQ) in ground water and surface water is 0.025 µg/L for BAS 720 H and its metabolites Reg. No. 4110603 and Reg. No. 4110542.

In this study, two parent-daughter ion transitions will be determined by LC-MS/MS. One transition will be used for evaluation, the other one for confirmation.

7.2 Extraction

Add 10 g of the water to a 15 mL centrifuge tube followed by the addition of 0.5 mL of 2 N HCl. For fortification experiments specimens are fortified at this step.

7.3 Conditioning of C_{18} SPE columns

Attach a Varian Bond Elut C-18 column on to a vacuum manifold by washing the column subsequently with 3 mL of each of the following solutions: hexane, DCM, methanol, water and 0.01 N HCl.

In a separate vacuum manifold, prepare the SCX column by eluting 3 mL of methanol through the column. Fill the column with 2 mL of methanol and set aside.

7.4 C-18 SPE Column only

Load the whole acidified specimen extract from step 6.2 to the C-18 column carefully. Wash the column with 3 mL of water followed by 3 mL of hexane. Stop the column when the liquid level passes just below the frit. Discard the wash.
Elute the above C-18 column with 3 mL of DCM and collect into a 15 mL glass centrifuge vial. Evaporate the DCM eluent at 40°C with a gentle nitrogen stream. Save the glass vial.

7.5 C-18 column couples with SCX SPE column

Attach the C-18 column to the top of the SCX column (still containing the 2 mL of methanol from step 6.3)
Add additional 3 mL of methanol to the top of C-18 column and eluate. Detach and discard the C-18 column from the SCX column.

7.6 SCX SPE column only

Wash the SCX column with 3 mL of methanol and discard the wash.
Elute the SCX column with 6 mL of water/methanol (20:80, v/v) into the same glass vial used to collect the eluent from the C1-8 column. Swirl the solvent to combine and mix with a vortex.

7.7 Sample preparation for Analysis

Evaporate the combined eluent from step 6.6 using a nitrogen evaporator with the water bath set to 70°C until dryness.
Reconstitute once completely dry, all specimens in 0.1 % HCOOH in water/methanol (80/20, v/v). Vortex to obtain an homogeneous solution. The specimens are ready for analyses.

Final determination was performed by LC-MS/MS monitoring two parent daughter ion transitions. One transition was used for evaluation, the other one for confirmation.

7.8 Equipment and Materials

7.8.1 General Laboratory Equipment

- shaking machine	Model SM 25 Bühler
- centrifuge	Model Heraeus Megafuge R16, Thermo Fisher
- analytical balance	Sartorius, model CPA225D-OCE
- top loading balance	Sartorius, model CPA 32025 + GS 3200-2
- ultra sonic bath	Bandelin, model DK 102
- vortex	Model reax 2000, Heidolph Heidolph, model 11
- nitrogen evaporator	Model 112 Organomation
- drying oven	Modle FD 115, WTB Binder
- ultra pure water unit	Millipore, model Synergy UV
- centrifuge tubes	Neolab, PP, 50 mL and 15 mL
- test tube for GPC sampler	glass 100x18x1 mm, with perforated PE-stopper, Burdich
- Pasteur pipette	glass
- volumetric pipette	different sizes
- volumetric flask	different sizes
- beaker	different sizes
- glass bottle	different sizes
- graduated cylinder	different sizes
- Dessicator	
- SPE manifold	Supelco
- injection vial, 1.8 mL, with PTFE sealed crimp-on caps	Burdich GmbH
- microvial	500 µL fitting into the injection vial
- sample vial	20 mL, Burdich
- adapter	
- mciropipettes	

7.8.2 Reagents

- dichloromethane	Roth, No. T162.1
- methanol	LGC Promochem SO-3041-B025
- ultra pure water	Millipore, Synergy UV
- sodium hydroxide	VWR, No. 1.06498
- formic acid	Merck 1.00264
- 1 mol/L Titrisol	Merck
- C_{18} SPE cartridges (0.2 g, 3 mL)	Bond Elute Varian, No. 12102025
- SCX SPE cartridges	Isolute / Biotage 530-0020-B

7.8.3 Preparation of the Mixtures

2 mol/L HCl	1 mol HCl (Titrisol) in 500 mL ultrapure water
Washing solution 5 C-18 clean-up 0.01 mol/L HCl	5 mL 2 mol/L HCl in 1 L ultrapure water
Elution solvent	200 mL ultrapure water with 800 mL methanol
SCX SPE clean-up	
0.1 % HCOOH in ultra pure water	1 mL conc. formic acid in 1 L water
Diluent	800 mL 0.1 % HCOOH in water with 200 mL methanol
Mobile Phase I	1 mL conc. formic acid in 1 L ultrapure water
MobilePhase II	1 mL conc. formic acid in 1 L methanol

7.8.4 Final Determination of BAS 720 H, Reg. No. 4110603 and Reg. No. 4110542

The residues of BAS 720 H and its metabolites were determined using LC-MS/MS equipment. The reported LC-MS/MS conditions are general. They may vary as they were adapted in order to obtain appropriate detector sensitivity and peak separation.

HPLC System:

System:	Agilent, No. 1200
Degasser:	Agilent, G1322A
Pump:	Agilent, G1311A
Injection system:	CTC Analytics, HTC Pal
Column oven with 6 port valve:	Agilent, G1316A
Control module:	Agilent, G4208A

HPLC-Conditions:

Analytical Column:	Zorbax Eclipse XDB-C18
length:	50 mm
interior diameter:	4.6 mm
particle size:	1.8 µm
Manufacturer:	Agilent

Oven temperature:	40 °C

Injection volume: 20 µL
Temperature of sample thermostat: 10 °C
Split (detector/waste): ---

Mobile Phase I: 0.1 % formic acid in water
Mobile Phase II: 0.1 % formic acid in methanol
Flow rate: see table below
Stop time: 15 minutes

Gradient time table:

Time [min]	Mobile Phase I [%]	Mobile Phase II [%]	Flow [mL/min]
0	80	20	0.6
0.5	80	20	0.6
3.0	50	50	0.6
7.0	5	95	0.6
7.10	5	95	0.6
10.0	5	95	0.6
10.1	80	20	0.6
15.0	80	20	0.6

Switching times for valve in column oven: 0 - 3.5 min (waste)
3.5 - 9.5 min (LC-MS/MS)
9.5 - 15 min (waste)

Retention times:
BAS 720 H: 5.2 min
Reg. No.4110603: 7.1 min
Reg. No. 4110542: 4.7 min

LC-MS/MS System:

System: Applied Biosystems, API 4000
Vacuum pump: HS602
Data system: Applied Biosystems, Analyst, version 1.5.1

LC-MS/MS Conditions:

		BAS 720 H
Scan type:	MRM	MRM
Polarity:	positive	positive
Curtain gas:	10	10
Collision gas:	8	8
Ionization voltage:	5500	5500
Temperature:	500	500
Entrance potential:	10	10
Declustering potential:	86	86
Collision energy:	31	43
Collision cell exit potential:	14	16
Transition used for evaluation:	306.3 → 261.2 (Quantification)	306.3 → 86.3 (Confirmation)

	Reg. No. 4110603	
Scan type:	MRM	MRM
Polarity:	positive	positive
Curtain gas:	10	10
Collision gas:	8	8
Ionization voltage:	5500	5500
Temperature:	500	500
Entrance potential:	10	10
Declustering potential:	96	96
Collision energy:	29	41
Collision cell exit potential:	16	10
Transition used for evaluation:	278.1 → 233.1 (Quantification)	278.1 → 165.0 (Confirmation)

	Reg. No. 4110542	
Scan type:	MRM	MRM
Polarity:	positive	positive
Curtain gas:	10	10
Collision gas:	8	8
Ionization voltage:	5500	5500
Temperature:	500	500
Entrance potential:	10	10
Declustering potential:	71	71
Collision energy:	31	27
Collision cell exit potential:	16	6
Transition used for evaluation:	306.3 → 261.2 (Quantification)	306.3 → 264.2 (Confirmation)

7.9 Preparation of Solutions for Fortification and Calibration

Immediately after preparation, the stock, fortification and calibration solutions were stored in capped vials protected from light in a refrigerator at 2 – 8 °C.

7.9.1 Stock and Fortification Solutions

Three stock solutions of BAS 720 H, Reg. No 4110603 and Reg. No 4110542 were prepared on 26 February 2013.*

Example:
On 26 February 2013, a stock solution of BAS 720 H, Reg. No 4110603 and Reg. No. 4110542 were prepared each in a volumetric flask by dissolving 11.07 mg in Methanol, 10.58 mg in Methanol and 11.89 mg in Methanol. This resulted in a concentration of 1.101 mg/mL, 1.039 mg/mL and 1.051 mg/mL taking into account a purity of 99.5 %, 98.2 and 88.4 % respectively.

* The stock solutions of BAS 720 H, Reg. No. 4110603 and Reg. No. 4110542 were prepared on 26 February 2013 and used for the BASF study 391104_1. These same stock solutions were simultaneously used for the study 391110.

The stock solution prepared on 26 February 2013 was dissolved daily fresh on 21 March 2013 with methanol to concentrations of 10.06 to 0.2012 µg/mL for BAS 720 H, 10.04 to 0.2008 µg/mL for Reg. No. 4110603 and 10.02 to 0.2004 µg/mL for Reg. No. 4110542 which were used for fortification.

These were used for the preparation of calibration solutions and fortification of the specimens.

7.9.2 Calibration Solutions

Example:
On 26 February 2013,a diluted stock solution of BAS 720 H, Reg. No. 4110603 and Reg. No 4110542 (concentration: 1.006, 1.004 and 1.002 µg/mL respectively) were dissolved in a 0.1 % HCOOH in H_2O/MeOH (80/20, v/v) solution to concentrations ranging from 0.0503 ng/mL to 2.515 ng/mL (BAS 720 H), 0.0502 to 2.515 ng/mL (Reg. No. 4110603) and 0.0501 to 2.505 ng/mL (Reg. No. 4110542) which were prepared for calibration.

No matrix matched calibration standards were necessary and therefore not used in this study.

Comparison between solvent standards and matrix-matched standard solutions

A solvent standard was compared against a matrix-matched standard for all analytes, Results are presented in the following table:

Analyte	**Imazamox**	**Imazamox**	**Reg. No. 4110603**	**Reg. No. 4110603**	**Reg. No. 4110542**	**Reg. No. 4110542**
Mass transition m/z	306 > 261	306 > 86	278 > 233	278 > 165	306 > 261	306 > 264
C std [ng/mL]	0.2515	0.2515	0.2505	0.2505	0.2510	0.2510
Peak area Solvent	6381	2649	3165	1721	9099	3003
Matrix	**surface water**					
Peak area Matrix	6227	3038	3639	1668	9173	3050
Signal Enhancement/ Suppresion by the matrix for the analyte	-2	15	15	-3	1	2
Matrix	**ground water**					
Peak area Matrix	7042	2826	3326	1522	8966	2877
Signal Enhancement/ Suppresion by the matrix for the analyte	10	7	6	-12	-1	-4

*A negative value indicates signal suppression, while a positive value means signal enhancement

7.10 Calculations

The detector signals were registered and integrated using the data system outlined in chapter 6.8.4. The peak area was taken into account to determine the BAS 720 H, Reg. No. 4110603 and Reg. No 4110542 amount in the specimens. The calibration curves were calculated from the area of the calibration solutions with their corresponding concentrations of the analytes using equation (1).

(1) $y = a + bx$

where
y: peak area [integration units iu]
x: amount of analyte [ng]
a: ordinate intercept [iu]
b: slope [iu/ng]

The amount of BAS 720 H, Reg. No. 4110603 and Reg. No. 4110542 in the specimen was calculated using the transformed equation (1):

(2) $$x = \frac{y - a}{b}$$

The concentration C of BAS 720 H, Reg. No. 4110603 and Reg. No. 4110542 in the specimen was calculated from x using equation (3):

(3) $$C = \frac{x \cdot V_E \cdot A_1}{V_i \cdot A_2 \cdot W}$$

where
x : amount of analyte [ng]
C : analysed concentration of the respective analyte in the specimen [µg/L]
V_E : final volume (1 and 5 mL)
A_1 : total extract (10 mL)
V_i : injection volume (20 µL)
A_2 : aliquot (10 mL)
W : specimen weight (for 10 mL)

The recovery data was calculated according to equation (4):

(4) $$R = \frac{C \cdot 100}{C_{for}}$$

where
R : recovery [%]
C : analysed concentration of the respective analyte in the fortified specimen
C_{for}: nominal concentration of respective analyte in the fortified specimen

7.11 Example for Calculation

The fortified specimen of ground water **130206571-A** was extracted and analysed on 21 March 2013. The parameters of the calibration curve for BAS 720 H on the basis of the detected mass transition m/z 306 → 261 was a = 6.923 (ordinate intercept), b = 1455807.07 derived from equation (1). The peak area acquired of the analyte BAS 720 H corresponded to 6702 counts.

The amount of BAS 720 H was calculated according to equation (2):

$$x = \frac{6702 - 6.923}{1455807} = 0.0046 \text{ [ng]}$$

The residue concentration was calculated according to equation (3):

$$C_R = \frac{0.0046 \cdot 10 \cdot 1}{10 \cdot 1 \cdot 20} = 0.023 \left[\frac{\mu g}{L}\right]$$

where
x: 0.0046 [ng]
C_R: analysed concentration of the respective analyte [µg/L]
V_E: 1 [mL]
A_1: 10 [mL]
V_i: 20 [µL]
A_2: 10 [mL]
W: 10 [mL]

The recovery data were calculated according to equation (4).

$$R = \frac{0.023 \cdot 100}{0.025} = 91\% \qquad (4)$$

where
R: recovery rate [%]
C_R: 0.023 [µg/L]
C_F: 0.025 [µg/L]

The tabulated values were rounded values based on calculations with the exact raw data.

The difference between the old and the fresh stock solutions of BAS 720 H was 3.4 %, proving that solutions of the reference item were stable for the duration of the laboratory work.

Stability of the Reference Item Reg. No. 4110603 during the Study (m/z 278→ 233)

Fresh Stock Standard Solution			Old Stock Standard Solution			
Stock Solution prepared on		26 Mar 2013	Stock Solution prepared on		26 Feb 2013	
Standard Solution diluted on		26 Mar 2013	Standard Solution diluted on		26 Mar 2013	
The date of analysis was 26 Mar 2013						
Concentration [ng/mL]	Peak Area	Quotient	Concentration [ng/mL]	Peak Area	Quotient	Percentage [%]
1.002	36169	36097	1.004	36788	36641	101.5

The difference between the old and the fresh stock solutions of Reg. No. 4110603 was 1.5 %, proving that solutions of the reference item were stable for the duration of the laboratory work.

Stability of the Reference Item Reg. No. 4110542 during the Study (m/z 306→ 261)

Fresh Stock Standard Solution			Old Stock Standard Solution			
Stock Solution prepared on		26 Mar 2013	Stock Solution prepared on		26 Feb 2013	
Standard Solution diluted on		26 Mar 2013	Standard Solution diluted on		26 Mar 2013	
The date of analysis was 26 Mar 2013						
Concentration [ng/mL]	Peak Area	Quotient	Concentration [ng/mL]	Peak Area	Quotient	Percentage [%]
1.004	13349	13296	1.002	13010	12984	97.7

The difference between the old and the fresh stock solutions of Reg. No. 4110542 was 2.3 %, proving that solutions of the reference item were stable for the duration of the laboratory work.

Since no matrix-matched standards were used within this study, these were not tested for stability during laboratory work.

8.7 Stability tests with Specimen extracts

In order to test the stability of the reference items in the final extracts (after clean-up treatments), an aliquot from these specimens fortified at 10times LOQ of the matrices ground water and surface water were diluted and analysed after 7 days. All stability analyses were determined using the quantification and confirmation ion transitions for all substances.
Storage temperature of the final specimen extracts: 2-8 °C
Storage period: 21.03.2013 (Extraction) to 28.03.2013 (Re-analyses)

11 Tables

Table 1: Detailed Information about the Specimen used for Fortification

Matrix	Date of		Specimen number
	Specimen received	Specimen homogenisation	SGS INSTITUT FRESENIUS
surface water	26 February 2013	n.a.	130160769
ground water	21 January 2013	n.a.	130206571

The specimen of surface water was stored at 2-8 °C and the specimen of ground water was stored deep frozen (≤ -18 °C) prior to extraction.

Table 2: Preparation of the Fortified Specimens

ground water

IF-Specimen No.	Weight of Specimen [g]	Volume of Fortification Solution [mL]	Concentration of Fortification Solution Imazamox [µg/L]	Concentration of Fortification Solution Reg. No. 4110603 [µg/L]	Concentration of Fortification Solution Reg. No. 4110542 [µg/L]	Date of Specimen Preparation
130206571-1	10.05	---	---	---	---	21-Mar-2013
130206571-2	10.00	---	---	---	---	21-Mar-2013
130206571-A	10.00	0.125	0.025	0.025	0.025	21-Mar-2013
130206571-B	10.00	0.125	0.025	0.025	0.025	21-Mar-2013
130206571-C	10.02	0.125	0.025	0.025	0.025	21-Mar-2013
130206571-D	10.01	0.125	0.025	0.025	0.025	21-Mar-2013
130206571-E	10.00	0.125	0.025	0.025	0.025	21-Mar-2013
130206571-F	10.01	0.125	0.25	0.25	0.25	21-Mar-2013
130206571-G	10.01	0.125	0.25	0.25	0.25	21-Mar-2013
130206571-H	10.04	0.125	0.25	0.25	0.25	21-Mar-2013
130206571-K	10.02	0.125	0.25	0.25	0.25	21-Mar-2013
130206571-L	10.03	0.125	0.25	0.25	0.25	21-Mar-2013

surface water

IF-Specimen No.	Weight of Specimen [g]	Volume of Fortification Solution [mL]	Concentration of Fortification Solution Imazamox [µg/L]	Concentration of Fortification Solution Reg. No. 4110603 [µg/L]	Concentration of Fortification Solution Reg. No. 4110542 [µg/L]	Date of Specimen Preparation
130160769-1	10.00	---	---	---	---	21-Mar-2013
130160769-2	10.02	---	---	---	---	21-Mar-2013
130160769-A	10.01	0.125	0.025	0.025	0.025	21-Mar-2013
130160769-B	10.01	0.125	0.025	0.025	0.025	21-Mar-2013
130160769-C	10.00	0.125	0.025	0.025	0.025	21-Mar-2013
130160769-D	10.00	0.125	0.025	0.025	0.025	21-Mar-2013
130160769-E	10.02	0.125	0.025	0.025	0.025	21-Mar-2013
130160769-F	10.00	0.125	0.25	0.25	0.25	21-Mar-2013
130160769-G	10.00	0.125	0.25	0.25	0.25	21-Mar-2013
130160769-H	10.02	0.125	0.25	0.25	0.25	21-Mar-2013
130160769-K	10.00	0.125	0.25	0.25	0.25	21-Mar-2013
130160769-L	10.01	0.125	0.25	0.25	0.25	21-Mar-2013

Table 3: Water Parameters

Parameter Determined	Result
surface water (130160769)	
temperature	4.9 °C
Redox potential	308 mV
Conductivity	82 µS/cm
Water hardness	0.0169 mmol/L
pH value	7.81
Dissolved organic Carbon (DOC)	0.931 mg/L
ground water (130206571)	
temperature	6.4 °C
Redox potential	252 mV
Conductivity	45 µS/cm
Water hardness	0.0049 mmol/L
pH value	6.00
Dissolved organic Carbon (DOC)	0.116 mg/L