2 Objective

The objective of this study was to demonstrate that method GS-004-W06-01 ("Analytical Method for the Determination of BYH18636 and its Metabolites BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide carboxylic acid, BYH18636-MMT and BYH18636-dicarboxy sulfonamide in Water Using LC/MS/MS") can be performed with acceptable recoveries for determination of the compound BYH18636 and its metabolites at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience Environmental Chemistry, Stilwell, USA, and reported as Method GS-004-W06-01, by Jami M. Wade & Derek J. Netzband, dated December 20, 2006. River Rhine water (taken at Leverkusen-Hitdorf, Germany) and tap water Monheim, Germany were chosen as representative matrices for validation within the present study.

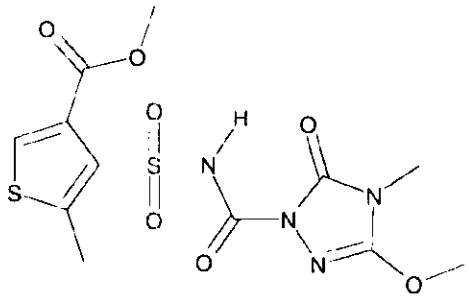
This study was performed in accordance with US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996.

3 Materials

3.1 Test and Reference Items

Code Name: BYH18636-a. i.

Chemical Name:	Methyl 4-[[[(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
Empirical formula:	$C_{12}H_{14}N_4O_7S_2$
Structural formula:	



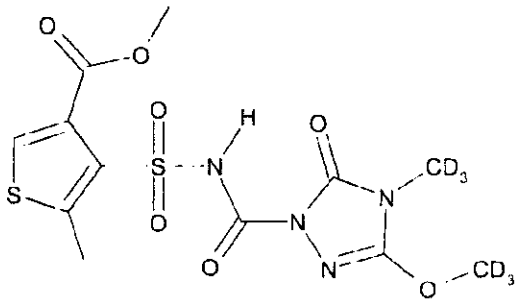
CAS Number	317815-83-1
Molecular weight:	390.4 g/mol
Certificate of analysis:	AZ 13227
Origin Batch No.:	GUE5969-2
Purity:	99.2%
Expiry date:	2008-03-06
Appearance:	white crystalline powder

Code Name: **BYH 18636-triazolinone-dimethyl-d_6**
(isotopic internal standard)

Chemical Name: Methyl 4-[[[[4,5-dihydro-3-(methoxy-d3)-4-(methyl-d3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate

Molecular Formula: $C_{12}H_8D_6N_4O_7S_2$

Structural formula:



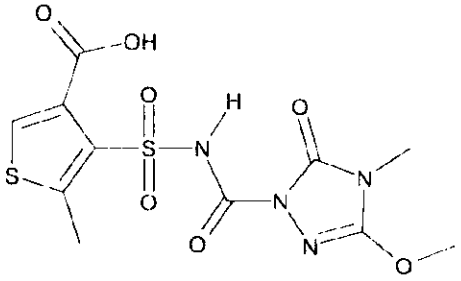
Molecular Weight: 396.43
Certificate of analysis: K-1362
Reference ID: 2003BRP176-249
Appearance: white solid

Code Name: **BYH18636-carboxylic acid**

Chemical Name: 4-[[[(4,5-Dihydro-3-methoxy-4-methyl-5-oxo-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid

Molecular Formula: $C_{11}H_{12}N_4O_7S_2$

Structural formula:



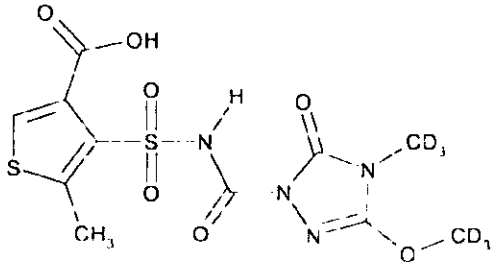
Molecular Weight: 376.37
Certificate of analysis: AZ 13393
Batch ID: GSE29091-6-1
Purity: 98.2%
Expiry date: 2009-05-18
Appearance: white powder

Code Name: **BYH 18636 Acid-triazolinone-dimethyl-d_6**
(Isotopic Internal Standard)

Chemical Name: 4-[[[[4,5-Dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid

Molecular Formula: $C_{11}H_6D_6N_4O_7S_2$

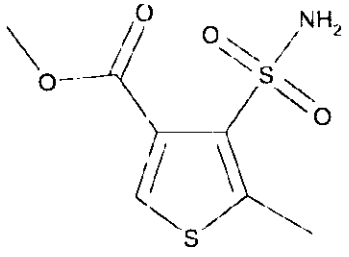
Structural formula:



Molecular Weight: 382.40
Certificate of analysis: K-1363
Reference ID: 2003BRP176-251
Appearance: white solid

Code Name: **BYH 18636 Sulfonamide**

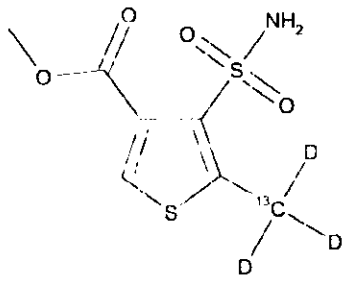
Chemical Name: Methyl 4-(aminosulfonyl)-5-methyl-3-thiophenecarboxylate
Molecular Formula: $C_7H_9NO_4S_2$
Structural formula:



CAS Number: 317815-81-9
Molecular Weight: 235.28
Certificate of analysis: AZ 13283
Batch ID: GUE5917-1
Purity: 99.3%
Expiry date: 2009-03-06

Code Name: **BYH 18636 Sulfonamide-5-methyl-13C d_3**
(Isotopic Internal Standard)

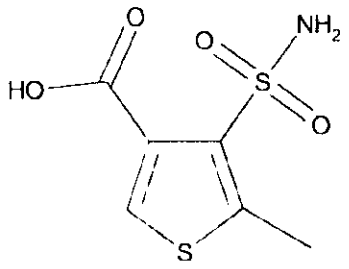
Chemical Name: Methyl-4-(aminosulfonyl)-5-(methyl-^{13}C-d_3)-3-thiophenecarboxylate
Molecular Formula: $C_7H_6D_3NO_4S_2$
Structural formula:



Molecular Weight: 239.30
Certificate of analysis: K-1441
Reference ID: 0213200601
Purity: 100%
Expiry date: 2016-10-02

Code Name: **BYH 18636 Sulfonamide Carboxylic acid**

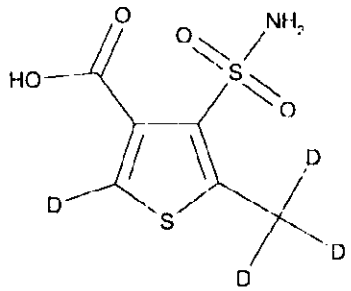
Chemical Name: 4-(Aminosulfonyl)-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_6H_7NO_4S_2$
Structural formula:



Molecular Weight: 221.25
Certificate of analysis: AZ 13160
Batch ID: GSE28269-1-1
Purity: 99.8%
Expiry date: 2009-01-19
Appearance: white powder

Code Name: **BYH 18636 Sulfonamide Carboxylic acid-2-d-methyl-d_3**
(Isotopic Internal Standard)

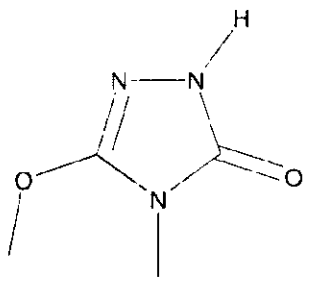
Chemical Name: 4-(Aminosulfonyl)-5-(methyl-d_3)-3-thiophene-2-d-carboxylic acid
Molecular Formula: $C_6H_3D_4NO_4S_2$
Structural formula:



Molecular Weight: 225.28
Certificate of analysis: K-1442
Reference ID: 0213200602
Purity: 100%
Expiry date: 2016-02-10

Code Name: **BYH 18636-MMT**

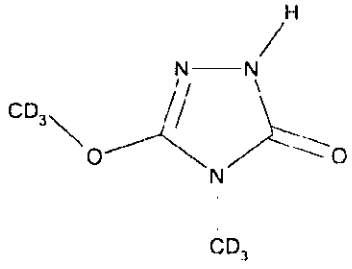
Chemical Name: 2,4-Dihydro-5-methoxy-4-methyl-3H-1,2,4-triazol-3-one
Molecular Formula: $C_4H_7N_3O_2$
Structural formula:



CAS Number: 135302-13-5
Molecular Weight: 129.12
Certificate of analysis: AZ 12554
Batch ID: GSE12201-6-5
Purity: 99.1%
Expiry date: 2007-05-02
Appearance: white powder

Code Name: **BYH 18636 Triazolinone-dimethyl-d_6**
(Isotopic Internal Standard)

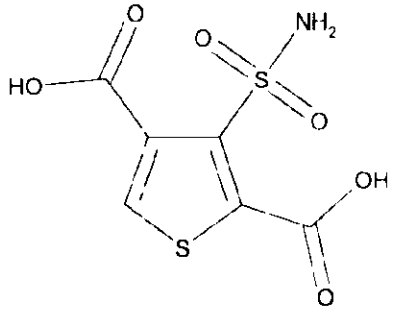
Chemical Name: 2,4-Dihydro-5-(methoxy-d_3)-4-(methyl-d_3)-3H-1,2,4-triazol-3-one
Molecular Formula: $C_4HD_6N_3O_2$
Structural formula:



Molecular Weight: 135.15
Certificate of analysis: K-1361
Reference ID: 2003BRP176-246
Purity: 95.9%
Expiry date: 2009-09-13

Code Name: **BYH 18636 Dicarboxy Sulfonamide**

Chemical Name: 3-(Aminosulfonyl)thiophene-2,4-dicarboxylic acid
Molecular Formula: $C_6H_5NO_6S_2$
Structural formula:



Molecular Weight: 251.24
Certificate of analysis: AZ 12698
Purity: 92%
Expiry date: 2007-05-31
Appearance: beige powder

3.2 Test System

The method was validated using River Rhine water and tap water Monheim. Two different water types were used in order to assess a possible influence of different water characteristics. The water samples were analysed for TOC, DOC, conductivity, water hardness, dry residue after filtration and pH by Bayer Industry Services, BIS SUA-PUA1, Building W15. Water types are summarised in Table 1.
Complete water parameters are reported in Table 13 and Table 14.

Table 1: Water Types

Water Type	Source of Water
Surface Water	River Rhine Water
Tap Water	Tap Water Monheim

4 Experimental

4.1 Analytical Method

The recovery data for the study were generated using the following method, which gives full details of preparing the analytical sample extracts and the conditions for high performance liquid chromatography (HPLC):

Number of the method:	GS-004-W06-01
Title of the method:	Analytical Method for the Determination of BYH18636 and its Metabolites BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide carboxylic acid, BYH18636-MMT and BYH18636-dicarboxy sulfonamide in Water Using LC/MS/MS
Author of the method:	Jami M. Wade & Derek J. Netzband Bayer CropScience Environmental Chemistry 17745 South Metcalf Avenue Stilwell, Kansas 66085
Reference:	Method GS-004-W06-01
Limit of quantitation:	0.5 µg/L

The following sample sets were analysed:

Table 2: Level and Number of Recoveries per Fortification Level

Water	Control sample	Level 0.5 µg/L	Level 5 µg/L
River Rhine	2	5	5
Tap Water Monheim	2	5	5

4.1.1 Outline of the Method

Appropriate volumes of fortification and internal standard solutions were added to the water samples followed by addition of formic acid and acetonitrile. After mixing the analysis was performed by LC-MS/MS.

4.1.2 Instruments

Liquid Chromatograph:	HP 1100 Column Compartment G1316A HP 1100 Binary Pump G1312A HP 1100 Isocratic Pump G1310A HP 1100 Degasser G1322A Agilent, 40880 Ratingen, Germany
Autosampler:	HTC PAL System CTC Analytics AG 4222 Zwingen, Switzerland
Mass Spectrometer:	Applies Biosystems API 4000 with turbo-ionspray interface mass selective detector (MS/MS) Note: Some mass spectrometric conditions are instrument specific. The spectrometric conditions were optimised by a competent operator prior to analysis.

4.1.3 Reagents and Equipment

	Column (HPLC): two LiChrospher 60 RP-select B, 5µm, length 125mm, i.d. 3 mm, coupled Series No.: Cat. 1. 50158, Merck KGaA, 64271 Darmstadt, Germany
Acetonitrile:	for HPLC, super gradient grade Riedel de Haën, No. 34998 30926 Seelze, Germany
Acetic acid (100%):	p.a. Merck, No.1.00063.1011 64271 Darmstadt, Germany
Water:	purified in a Milli-Q unit Milli-Pore GmbH 65731 Eschborn, Germany

Volumetric flasks, pipettes and other equipment commonly used in the laboratory.

4.1.4 Liquid Chromatographic Conditions

Liquid chromatographic conditions were from those described in Appendix 1 of the original method report GS-004-W06-01. The HPLC gradient program had to be adjusted to the used equipment. In addition acetic acid had to be substituted by formic acid in the mobile phase A. During the initial set of analyses low recoveries for one of the metabolites was obtained when using acetic acid in mobile phase, however it was determined that acceptable recoveries were obtained when substituting formic acid for acetic acid, as described in Section 6.

Table 3: Liquid Chromatographic Conditions

Column:	Column (HPLC): two LiChrospher 60 RP-select B, 5µm, length 125mm, i.d. 3mm, coupled
Particle size:	5 µm
Oven temperature:	40 °C
Injection volume:	60 µL
Run time:	21 minutes
Mobile phase:	A: milli-Q-water (+ 0.5% formic acid)
	B: acetonitrile (+ 0.1% formic acid)
Retention times:	approx. 13.1 min for BYH18636
	approx. 12.4 min BYH18636-carboxylic acid
	approx. 12.4 min BYH18636-sulfonamide
	approx. 11.4 min BYH18636-sulfonamide carboxylic acid
	approx. 8.1 min BYH18636-MMT
	approx. 5.7 min BYH18636-dicarboxy sulfonamide

Table 4: HPLC Gradient

Time [min]	Flow [mL]	A [%]	B [%]	
0.00	300	90.0	10.0	
0.10	300	90.0	10.0	
3.00	300	80.0	20.0	
4.50	300	75.0	25.0	*
5.20	300	75.0	25.0	*
9.00	300	60.0	40.0	
10.50	600	30.0	70.0	*
11.50	600	10.0	90.0	
15.45	300	10.0	90.0	
15.50	300	10.0	90.0	
15.51	300	99.0	1.0	
17.99	300	99.0	1.0	*
18.00	300	90.0	10.0	*
21.00	300	90.0	10.0	*

* changed with respect to the original method description

Table 5: Valco Valve Method Properties

Time [min]	
0.0	switch eluent stream into waste
4.0	switch eluent stream into interface
15.0	switch eluent stream into waste

These switching times were also adjusted to the used equipment.

4.1.5 Mass Spectrometric Parameters

MS/MS parameter settings were in general as described in method GS-004-W06-01 but optimized for the instrument being used.

4.1.6 Calculation

For calculation of the concentrations, seven-point calibration curves were used. These curves were calculated using linear regression automatically after each sequence run with the Perkin-Elmer quantitation software Analyst (vers. 1.4.1). Further calculations were performed using the software MS-EXCEL 2002. The results given are rounded values. Thus, rounding "errors" may occur if recalculations are made using the listed figures.

4.1.6.1 Calculation of Analyte Concentrations

NOTE: Evaluation is performed according to the linearity standard procedure.

1. Calculate the response factors (peak area of detected analyte / peak area of the internal standard) of all standard injections and calculate the resulting linearity of the analyte.
2. Determine the response factor (peak area of detected analyte / peak area of the internal standard) for the sample. This value will be used as x.
3. Calculate the residue level in µg/L as follows:

$$R = (x - b) / a \cdot Df$$

R: Determined concentration of analyte in µg/L
x: Response factor of the sample
b: Interception from linear regression
a: Slope from linear regression
Df Dilution factor (in this method 1.11)

4.1.6.2 Calculation of Recoveries

1. Calculate the concentration in the recovery sample according to 4.1.6.1.
2. Calculate the percent recovery as follows:

$$\text{Recovery} = \frac{\text{Concentration} \times 100}{\text{Fortification Level}}$$

Recovery:	Recovered concentration of analyte in % found in the fortified sample
Concentration:	Concentration in the fortified sample in µg/L determined according to 4.1.6.1
Fortification level:	Fortified concentration of analyte in µg/L

4.1.7 Deviations from the Method

Within the analytical procedure for determination of BYH18636 and its metabolites some details have been changed.
In order to obtain a sufficient separation of the peaks on the HPLC gradient program had to be adjusted to the used equipment. In addition acetic acid was substituted by formic acid in the mobile phase A.