2 Objective

The objective of this study was to demonstrate that method GS-003-S06-01 ("Analytical Method For The Determination of Residues of BYH18636 And Its Metabolites BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT, and BYH18636-triazolinone-carboxamide In Soil and Sediment Using LC/MS/MS") can be performed with acceptable recoveries for determination of the compounds BYH18636, BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT, and BYH18636-triazolinone-carboxamide at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience LP, Stilwell, USA, and validated in that laboratory with results reported as Method GS-003-S06-01, by D.J. Netzband & J.M. Wade, dated June 13, 2006. Soil Höfchen (Germany) and soil Laacher Hof (Germany) were chosen as representative matrices for validation within the present study.

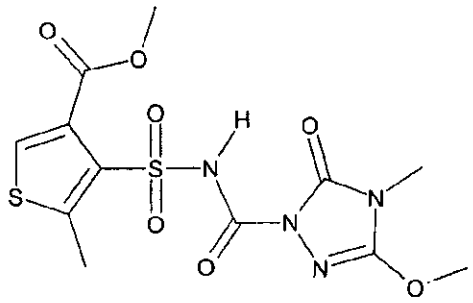
This study was performed in accordance with EC Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 of March 17, 2004, Commission Directive 96/46/EC amending Council Directive 91/414/EEC of July 16, 1996, BBA Guideline on Residue Analytical Methods for Post-Registration Control Purposes of July 21, 1998, US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996 and US EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue Analytical Method, EPA 712-C-96-174, August 1996.

3 Materials

3.1 Test and Reference Items

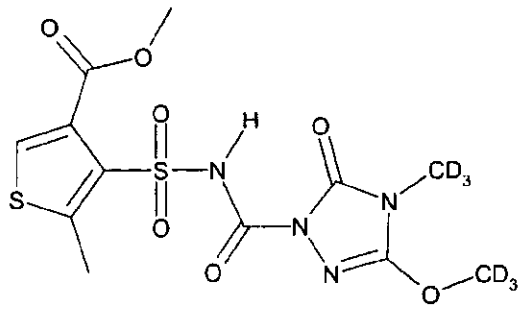
The structures for BYH18636, its metabolites and the associated internal standards are *presented below*:

Code Name: BYH18636 (AE 1162464)
(Parent Molecule)



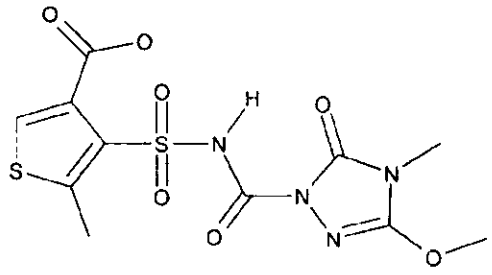
CAS Name: Methyl 4-[[[(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
CAS Number 317815-83-1
Molecular Formula: $C_{12}H_{14}N_4O_7S_2$
Molecular Weight: 390.40
Certificate of analysis: AZ 12208
Batch ID: NLL6954-10
Purity: 99.3%
Expiry date: 2007-01-17

Code Name: BYH18636-triazolinone-dimethyl-d6
(parent molecule, isotopic internal standard)



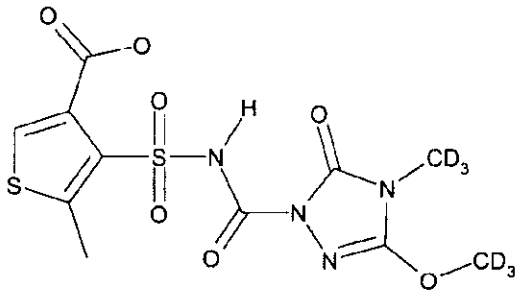
CAS Name: Methyl 4-[[[[4,5-dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1*H*-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
Molecular Formula: $C_{12}H_8D_6N_4O_7S_2$
Molecular Weight: 396.43
Certificate of analysis: K-1362
Reference ID: 2003BRP176-249

Code Name: BYH18636-carboxylic acid (AE 1394083)
(soil metabolite)



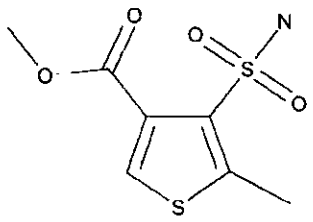
CAS Name: 4-[[[(4,5-Dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_{12}N_4O_7S_2$
Molecular Weight: 376.37
Certificate of analysis: AZ 13393
Batch ID: GSE29091-6-1
Purity: 98.2%
Expiry date: 2009-05-18

Code Name: BYH18636 Acid-triazolinone-dimethyl-d6
(soil metabolite, isotopic internal standard)



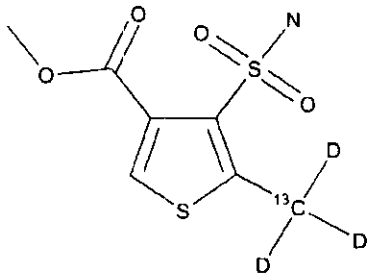
CAS Name: 4-[[[[4,5-Dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_6D_6N_4O_7S_2$
Molecular Weight: 382.40
Certificate of analysis: K-1363
Reference ID: 2003BRP176-251

Code Name: BYH18636-sulfonamide (AE 1364547)
(soil metabolite)



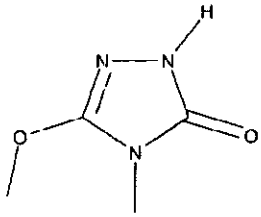
CAS Name: Methyl 4-(aminosulfonyl)-5-methyl-3-thiophenecarboxylate
CAS Number: 317815-81-9
Molecular Formula: $C_7H_9NO_4S_2$
Molecular Weight: 235.28
Certificate of analysis: AZ 13283
Batch ID: GUE5917-1
Purity: 99.3%
Expiry date: 2009-03-06

Code Name: Sulfonamide-5-methyl-$^{13}Cd_3$
(soil metabolite, isotopic internal standard)



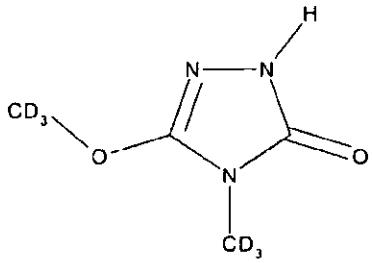
CAS Name: Methyl-4-(aminosulfonyl)-5-(methyl-^{13}C-d_3)-3-thiophenecarboxylate
Molecular Formula: $C_7H_6D_3NO_4S_2$
Molecular Weight: 239.30
Certificate of analysis: K-1441
Reference ID: 0213200601
Purity: 100%
Expiry date: 2016-10-02

Code Name: BYH18636-MMT (AE 1277106)
(soil metabolite)



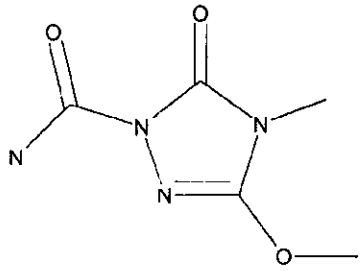
CAS Name: 2,4-Dihydro-5-methoxy-4-methyl-3*H*-1,2,4-triazol-3-one
CAS Number: 135302-13-5
Molecular Formula: $C_4H_7N_3O_2$
Molecular Weight: 129.12
Certificate of analysis: AZ 12554
Batch ID: GSE12201-6-5
Purity: 99.1%
Expiry date: 2007-05-02

Code Name: Triazolinone-dimethyl-d6
(soil metabolite, isotopic internal standard)



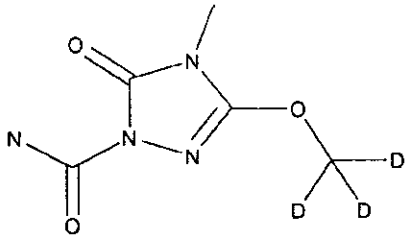
CAS Name: 2,4-Dihydro-5-(methoxy-d_3)-4-(methyl-d_3)-3H-1,2,4-triazol-3-one
Molecular Formula: $C_4HD_6N_3O_2$
Molecular Weight: 135.15
Certificate of analysis: K-1361
Reference ID: 2003BRP176-246
Purity: 95.9%
Expiry date: 2009-09-13

Code Name: BYH18636-triazolinone carboxamide (AE 1430601)
(soil metabolite)



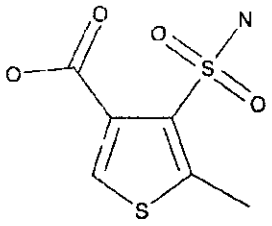
CAS Name: 4,5-Dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazole-1-carboxamide
Molecular Formula: $C_5H_8N_4O_3$
Molecular Weight: 172.14
Certificate of analysis: AZ 13494
Batch ID: GSE28097-2-1
Purity: 95.3%
Expiry date: 2009-07-03

Code Name: BYH18636-triazolinone-carboxamide-methoxy-d_3
(soil metabolite, isotopic internal standard)



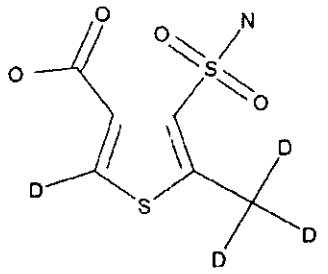
CAS Name: Unknown
Molecular Formula: $C_5H_5D_3N_4O_3$
Molecular Weight: 175.16
Certificate of analysis: K-1516
Reference ID: 2005BRP176-131
Purity: 96.3%
Expiry date: 2015-06-03

Code Name: BYH18636-sulfonamide-carboxylic acid (AE 1395853)
(soil metabolite)



CAS Name: 4-(Aminosulfonyl)-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_6H_7NO_4S_2$
Molecular Weight: 221.25
Certificate of analysis: AZ 13160
Batch ID: GSE28269-1-1
Purity: 99.8%
Expiry date: 2009-01-19

Code Name: Sulfonamide Carboxylic acid-2-d-methyl-d3
(soil metabolite, isotopic internal standard)



CAS Name: 4-(Aminosulfonyl)-5-(methyl-d_3)-3-thiophene-2-d-carboxylic acid

Molecular Formula: $C_6H_3D_4NO_4S_2$
Molecular Weight: 225.28
Certificate of analysis: K-1442
Reference ID: 0213200602
Purity: 100%
Expiry date: 2016-02-10

3.2 Test System

The method was validated using two German soils *Höfchen* and *Laacher Hof*. Two different soils were used in order to assess a possible influence of different soil properties. The soil samples were classified according to USDA specifications. Main characteristics of the used soils are summarised in Table 1.
Detailed physical/chemical soil parameterisation is reported in Table 11 and Table 12.

Table 1: Soil Types

Soil	Texture of Soil	Organic Matter [%]
Höfchen	silt loam (USDA)	1.58
Laacher Hof	sandy loam (USDA)	2.06

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-003-S06-01
Title of the method:	Analytical method for the determination of residues of BYH18636 and its metabolites BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT, and BYH18636-triazolinone-carboxamide in soil and sediment using LC/MS/MS, June 13, 2006
Authors of the method:	Derek J. Netzband and Jami M. Wade Bayer CropScience LP 17745 S Metcalf Avenue Stilwell, Kansas 66085, USA
Reference:	Method GS-003-S06-01
Limit of quantitation:	2 µg/kg

The following sample sets were analysed:

Table 2: Level and Number of Recoveries per Fortification Level

Soil	Control sample	Level 2 µg/kg	Level 20 µg/kg
Höfchen	2	5	5
Laacher Hof	2	5	5

Additionally, a solvent blank with internal standards added prior to LC-MS/MS analysis was included in the set.

4.1.1 Outline of the Method

BYH18636 and its associated metabolites are extracted from soil and sediment using microwave extraction. An isotopic internal standard solution containing BYH18636-triazolinone-dimethyl-d_6, BYH18636 acid-triazolinone-dimethyl-d_6, BYH18636-sulfonamide-$^{13}C,d_3$, Triazolinone-dimethyl-d_6, BYH18636-triazolinone-carboxamide-methoxy-d_3 and BYH18636 acid-sulfonamide-d_4 is added to the extract, which is acidified and partitioned with ethyl acetate. The ethyl acetate is evaporated to dryness, reconstituted in water/acetonitrile and analyzed by LC/MS/MS for BYH18636, BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT and BYH18636-triazolinone-carboxamide.

4.1.2 Instruments

Microwave Extractor:	MLS-Ethos MWS Vertriebs GmbH 88299 Leutkirch, Germany
Balance:	XP 603 S and XP 205 Mettler Instruments GmbH 35387 Giessen, Germany
Ultrasonic Bath:	Transsonic 890/H Heinrich Faust 51145 Cologne, Germany
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:	API 4000 with turbo-ionspray interface mass selective detector (MS/MS) Applied Biosystems 64331 Weiterstadt, Germany

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:	LiChrospher® 60 RP-select B 5µ 125 x 3.00 mm Column Part No. 1158920 Fisher Scientific GmbH 58239 Schwerte, Germany
Syring Filter:	Acrodisc 0.45µm 13mm Pall Life Sciences Part No. 4426T Fisher Scientific GmbH 58239 Schwerte, Germany
Magnetic stirring bar:	plain (large, e.g. 35 x 8 mm [length x i.d.]) or "dumb-bell" type (e.g. 35 x 8 mm [length x i.d.], diameter of end disk is 20 mm, from COWIE Technology, parts no. 1.1335) or equivalent
Acetonitrile:	for HPLC, super gradient grade Riedel de Haen, No. 34998 30926 Seelze, Germany
Acetic Acid:	p.a. Merck, No.1.00063.1011 64271 Darmstadt, Germany
Sodium sulphate:	Anhydrous >98.5% KMF No. 03-020.1000 53785 Lohmar, Germany
Formic acid (100%)	p.a. Merck, No.1.00264.2500 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 Chromatographic Conditions and Mass Spectrometric Parameters

MS/MS parameter settings were optimized for the instrument being used and therefore not identical with those reported in method GS-003-S06-01.

4.1.5 Calculation

Calculations were performed using the computer software MS-EXCEL. In general, the program uses nine decimal places for calculations. The results given are rounded values. Thus, rounding "errors" may occur if recalculations are made using the listed figures.

For calculation of the concentrations, calibration curves were used. These curves were calculated using linear regression automatically after each sequence run with the Applied Biosystems quantitation software Analyst (vers. 1.4). Further calculations were performed using the software Microsoft® EXCEL 2002. Matrix effects are eliminated by using an internal standard solution of the isotopically labelled reference substance.

The linear equation is expressed as:

$$y = \text{Intercept} + \text{Slope} \bullet x$$

y = Int. Ratio, x = Conc.Ratio

$$y = \frac{\text{Area}_{\text{Standard}}}{\text{Area}_{\text{Internal Standard}}} = \text{Int.Ratio} \quad \text{and} \quad x = \frac{\text{Conc}_{\text{Standard}}}{\text{Conc}_{\text{IS}}} = \text{Conc.Ratio}$$

Int.Ratio:	*intensity ratio*
ConcStandard:	*concentration of standard solution [µg/L]*
ConcIS:	*concentration of internal standard solution [µg/L]*
Conc.Ratio:	*concentration ratio*

Because the concentration of the isotopically labelled internal standards was the same in all solutions that were injected into the HPLC instrument, it has not to be taken into consideration. However, the concentration of the internal standard should be similar to the analyte concentration.
By means of the linear equation, the content of BYH18636, BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT and BYH18636-triazolinone-carboxamide in dry soil can be calculated as follows:

$$\text{Dilution}_{\text{Factor}} = \frac{\text{Volume}_{\text{Extraction}} \times \text{Volume}_{\text{End}}}{\text{Weight} \times \text{Volume}_{\text{Aliquot}}}$$

$$\text{Conc}_{\text{Analyte}} = \frac{\text{Int.Ratio} - \text{Intercept}}{\text{Slope}}$$

$$\text{Conc}_{\text{Soil Wet}} = \text{Conc}_{\text{Analyte}} \times \text{Dilution}_{\text{Factor}}$$

$$\text{Conc}_{\text{Soil Dry}} = \text{Conc}_{\text{Soil Wet}} \times \frac{100\%}{100\% - \text{Moisture}}$$

$Conc_{Analyte}$:	*concentration of the analyte in the sample solution [µg/L]*
$Conc_{Soil\ Wet}$:	*concentration of the analyte in wet soil [µg/kg]*
$Conc_{Soil\ Dry}$:	*concentration of the analyte in dry soil [µg/kg]*
Intercept:	*intercept of the linear regression curve*
Slope:	*slope of the linear regression curve [L/µg]*
Volume:	*volumen of the extraction solvent, the aliquot and final solution [L]*
Weight:	*weight of the soil sample [kg]*

The recovery is calculated according to the following equation:

$$\text{Recovery} = \frac{Conc_{Soil\ Wet} \times 100\%}{Conc_{Soil\ Spiked}}$$

$Conc_{Soil\ Spiked}$: *concentration of the spiked reference substance [µg/kg]*

4.1.6 Deviations from the Method

According to method GS-003-S06-01 the samples are extracted in the microwave using the following program (page 16 of the method report):

Time:	6 minutes
Temperature:	50 °C
Watts:	350 max

During establishment of the method it became apparent that the given information for the microwave program can be interpreted in different ways, e.g. within 6 min to 50 °C, using a maximum of 350 W. However, in direct correspondence with the lab which developed the method (see section 6 of this report), it was stated, that for acceptable extraction efficiency the samples need to be exposed to 50 °C for 3-4 minutes. For this reason the temperature program should be interpreted as follows: Samples should be heated to 50 °C within the shortes possible time period using at 350 Watts. After reaching 50 °C this temperature must be maintained for the remainder of the 6 minute run time.

Note: When extracting a set of 7-8 samples at the same time it will last about 3 min until the max. temperature of 50 °C is reached. Accordingly, within this independent validation study no more than 7 samples were extracted in the microwave at the same time.

On page 15 of the method report (section 7.4) it is stated that calibration standard solutions are diluted with 99:1 v/v acetonitrile:deionized water. It must be the other way around, i.e. 1:99 v/v acetonitrile:deionized water, since the HPLC gradient starts with 1% acetonitrile.

On page 21, Table 1 the ratio for the extraction solvent must be changed to 35% acetonitrile/65% water to be consistent with the ratio given on page 16 (see also section 6 of this report).

On page 20 in section 10.4 of the method report a table with the primary and alternate ion transitions is given. The alternate ion transition of BYH18636-triazolinone-carboxamide of m/z 153 in Q3 could not be confirmed (no signal seen in the respective mass spectra of BYH18636-triazolinone-carboxamide). Furthermore, in the spectrum 6 of BYH18636-triazolinone-carboxamide on page 40 of the method report the mass peak

of 153 is seen, however the mol mass peak is m/z 171 instead of m/z 173. It can not be excluded that a wrong spectrum was incorporated in the report.

4.2 Linearity of the Detector

The linearity of the detector response for BYH18636 and its metabolites BYH18636-carboxylic acid, BYH18636-sulfonamide, BYH18636-sulfonamide-carboxylic acid, BYH18636-MMT and BYH18636-triazolinone-carboxamide were tested by injections of standard solutions. The following concentrations were measured:

Table 3: Standard Concentrations for the Determination of Detector Linearity

	Concentration [µg/L]					
BYH18636	1	2	5	10	20	40
BYH18636-carboxylic acid	1	2	5	10	20	40
BYH18636-sulfonamide	1	2	5	10	20	40
BYH18636-sulfonamide-carboxylic acid	1	2	5	10	20	40
BYH18636-MMT	1	2	5	10	20	40
BYH18636-triazolinone-carboxamide	1	2	5	10	20	40