2. BACKGROUND INFORMATION

The objective of this study was to validate a method of analysis for the determination of BYH 18636 and its associated metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636 sulfonamide-carboxylic acid, BYH 18636-MMT and BYH 18636-dicarboxy sulfonamide in water by LC/MS/MS.

On completion of this study the analytical method GS-004-W06-01: "Analytical Method For The Determination of Residues of BYH 18636 And Its Metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636 sulfonamide-carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide In Water Using LC/MS/MS" was issued.

An Independent Laboratory Validation Study (ILV) [6] was performed on analytical method GS-004-W06-01 (See Section 9.7). The analytical method was updated to include the data generated during the ILV, and the updated method was assigned an analytical method number of GS-004-W06-02. Method GS-004-W06-02 is presented in Appendix 3 of this report.

The study was performed in accordance with United States Environmental Protection Agency (EPA) Pesticide Assessment Guidelines and Good Laboratory Practices (and Ecological Effects Test Guidelines OPPTS 850.7100[1] and Residue Chemistry Test Guidelines, OPPTS 860.1340[2]). This validation fulfils the requirement that properly validated methods of analysis be utilized for the generation of pesticide residue data and for tolerance enforcement.

Nomenclature for BYH 18636 and its metabolites are presented in Section 4.

3. EXPERIMENTAL DESIGN

This study was conducted following an approved protocol. All amendments to the protocol were signed and dated by the Study Director and the Sponsor's Representative. Any deviations from the protocol were documented and brought to the Study Director's attention when they were noted and maintained with the raw data.

This study was initiated on November 21, 2006. The experimental phase of the study began on November 29, 2006 and concluded on December 5, 2006. The following personnel were involved in the conduct of this study:

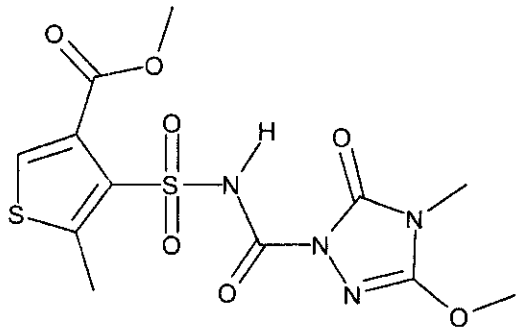
Jami Wade
R and D Specialist I
Environmental Chemistry

Derek J. Netzband
Senior Scientist
Environmental Chemistry

4. TEST AND REFERENCE SUBSTANCES

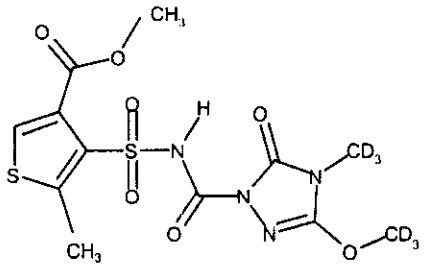
The following compounds were used as test and reference substances, and were supplied by Bayer CropScience. Neat standards were stored in a freezer at approximately –21°C. Standard solutions were stored in a refrigerator at approximately 5°C.

Code Name: BYH 18636 (Thiencarbazone-methyl or AE 1162464)
(Parent Molecule)



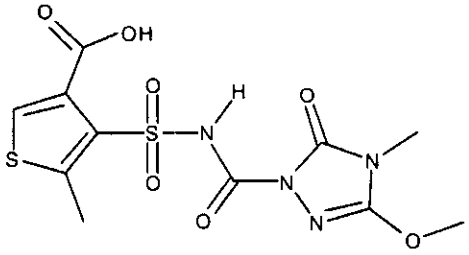
CAS Name:	Methyl 4-[[[(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
CAS Number	317815-83-1
Molecular Formula:	$C_{12}H_{14}N_4O_7S_2$
Molecular Weight:	390.3922
ID No.:	K-1439
Reference No.:	0124200503
Purity:	99.3%
Expiration Date:	01/17/07
Storage Conditions:	Frozen
Source:	Bayer CropScience, Kansas City, Missouri

Code Name: BYH 18636-triazolinone-dimethyl-d6
(Parent Molecule, Isotopic Internal Standard)



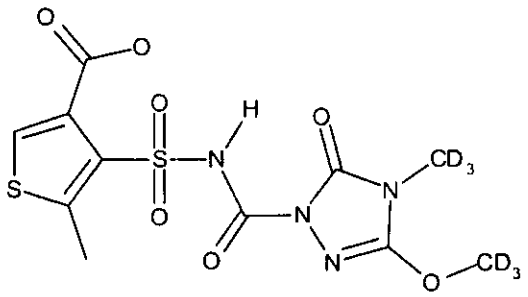
CAS Name: Methyl 4-[[[[4,5-dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1*H*-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
Molecular Formula: $C_{12}H_8D_6N_4O_7S_2$
Molecular Weight: 396.4291
ID No.: K-1362
Reference No.: 2003BRP176-249
Purity: 99.6%
Expiration Date: 8/11/14
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: BYH 18636-carboxylic acid (AE 1394083)
(Water Metabolite)



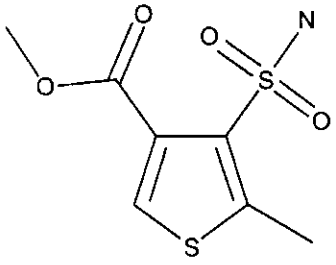
CAS Name: 4-[[[(4,5-Dihydro-3-methoxy-4-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_{12}N_4O_7S_2$
Molecular Weight: 376.3656
ID No.: K-1596
Reference No.: 0203200604
Purity: 98.2%
Expiration Date: 5/18/09
Storage Conditions: Frozen
Source: Bayer CropScience, Kansas City, Missouri

Code Name: BYH 18636 Acid-triazolinone-dimethyl-d6 (or d_6-AE 1394083)
(Water Metabolite, Isotopic Internal Standard)



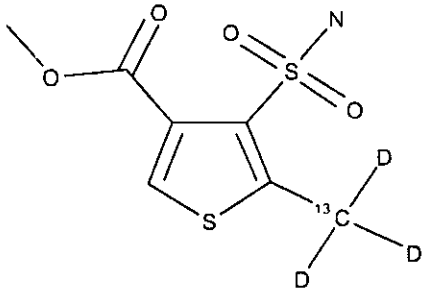
CAS Name: 4-[[[[4,5-Dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_6D_6N_4O_7S_2$
Molecular Weight: 382.4025
ID No.: K-1363
Ref No.: 0811200402
Purity: 99.7%
Expiration Date: 08/10/14
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: BYH 18636-sulfonamide
(Water Metabolite)



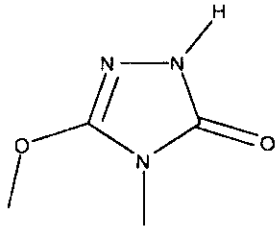
CAS Name: Methyl 4-(aminosulfonyl)-5-methyl-3-thiophenecarboxylate
CAS Number: 317815-81-9
Molecular Formula: $C_7H_9NO_4S_2$
Molecular Weight: 235.2807
ID No.: K-1550
Reference No.: 0210200501
Purity: 99.3%
Expiration Date: 3/6/09
Storage Conditions: Frozen
Source: Bayer CropScience, Kansas City, Missouri

Code Name: Sulfonamide -5-methyl-$^{13}Cd_3$ (or $^{13}C,d_3$-BYH 18636 Sulfonamide)
(Water Metabolite, Isotopic Internal Standard)



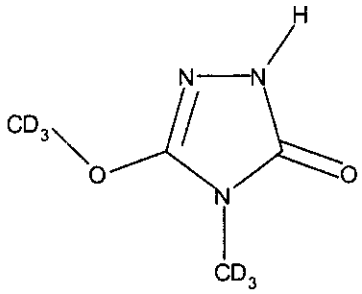
CAS Name: Methyl 4-(aminosulfonyl)-5-(methyl-^{13}C-d_3)-3-thiophenecarboxylate
Molecular Formula: $C_7H_6D_3NO_4S_2$
Molecular Weight: 239.3039
ID No.: K-1441
Ref. No.: 0213200601
Purity: 100%
Expiration Date: 02/10/16
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: BYH 18636-MMT (AE 1277106)
(Water Metabolite)



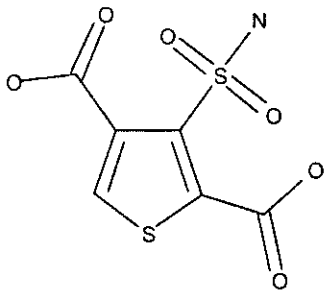
CAS Name: 2,4-Dihydro-5-methoxy-4-methyl-3*H*-1,2,4-triazol-3-one
CAS Number: 135302-13-5
Molecular Formula: $C_4H_7N_3O_2$
Molecular Weight: 129.1173
ID No.: K-1443
E.R. Ref. No.: 95R-31-150A
Purity: 87.8%
Expiration Date: 6/27/07
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: Triazolinone-dimethyl-d_6 (or d_6-MMT)
(Water Metabolite, Isotopic Internal Standard)



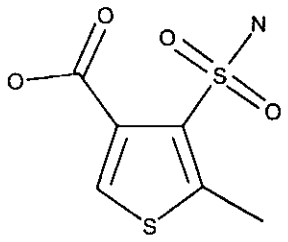
CAS Name: 2,4-Dihydro-5-(methoxy-d_3)-4-(methyl-d_3)-3H-1,2,4-triazol-3-one
Molecular Formula: $C_4HD_6N_3O_2$
Molecular Weight: 135.1542
ID No.: K-1361
E.R. Ref. No.: 2003BRP176-246
Purity: 95.9%
Expiration Date: 9/13/09
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: BYH 18636-dicarboxy sulfonamide (AE 1430601)
(Water Metabolite)



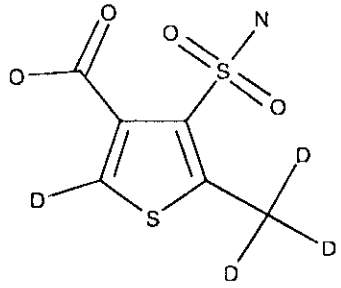
Molecular Formula: $C_6H_5NO_6S_2$
Molecular Weight: 251.2370
ID No.: K-1585
E.R. Ref. No.: 1212200501
Purity: 92%
Expiration Date: 5/31/07
Date of Analysis: 5/31/05
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: BYH 18636-sulfonamide-carboxylic acid (AE 1395853)
(Water Metabolite)



CAS Name: 4-(Aminosulfonyl)-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_6H_7NO_4S_2$
Molecular Weight: 221.2541
ID No.: K-1380
E.R. Ref. No.: 0615200405
Purity: 99.8%
Expiration Date: 1/19/09
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

Code Name: Sulfonamide Carboxylic acid-2-d-methyl-d_3 (or d_4-BYH 18636 Sulfonamide)
(Water 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: 225.2787
ID No.: K-1442
Ref. No.: 0213200602
Purity: 100%
Expiration Date: 2/10/16
Storage Conditions: Frozen
Source: Bayer CropScience, Stilwell, Kansas

5. TEST SYSTEM – WATER SAMPLES

The method was validated for surface raw water, ground water, and drinking water. The ground water used in this study was a composite collected in Monmouth County, New Jersey for Bayer CropScience Study Number BL212401[3]. The raw surface water used in this study was untreated control (UTC) raw water collected in Palm Beach County, Florida during Bayer CropScience Study Number RAOAY001[4]. Chlorinated HPLC water prepared in the analytical laboratory was used for the drinking water samples.

6. STORAGE

The untreated water samples were refrigerated.

7. REAGENTS AND EQUIPMENT

7.1 Reagents and General Equipment

The reagents and equipment used in this study are listed in Sections 5 and 6 of the method of analysis presented in Appendix 3.

Appropriate Material Safety Data Sheets were available to the study personnel during the conduct of the study. General laboratory safety precautions were taken.

7.2 Liquid Chromatographic/Mass Spectrometer Detection System

Residues of BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide-carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide in water were determined using an Applied Biosystems Sciex API-4000 LC/MS/MS system, Shimadzu LC-10AD VP HPLC pumps (2) with a high pressure mixer, a SCL-10A VP Pump Controller and a Perkin Elmer 200 Series autosampler. The Applied Biosystems instrument software applications used was Analyst 1.4.1.

The LC conditions used for the water validation and MS/MS operating parameters used are outlined in Appendix 1 of the analytical method presented in Appendix 3 of this report.

Representative chromatograms using these LC conditions are presented in Appendix 1 of this report.

8. CALCULATIONS

8.1 Calibration Curves

Standard concentrations of BYH 18636 and its metabolites typically ranged from 0ng/mL to 10.0ng/mL, each with 0.5ng/mL isotopic internal standard added. The calibration standards were interspersed with the samples. All calculations were performed using Applied Biosystems Analyst software (Version 1.4.1) or Microsoft® Excel worksheets. Linear regression coefficients were calculated for the ratio of

analyte to internal standard area plotted versus the area of analyte in the calibration standards.

8.2 Quantification of Residues

The calculation technique and an example calculation is presented in Appendix 2 of this report.

2. BACKGROUND

The herbicide BYH 18636 is currently being developed by Bayer CropScience.

An analytical method was developed for the analysis of BYH 18636 and its associated metabolites in water and the method was validated in Bayer CropScience Study Number RAGSM001[1].

The structures for these compounds are presented in Section 4. This analytical method was prepared based on the results obtained in the validation study.

Typical recovery results are presented in Appendix 3, and the data shown was obtained from the method validation study.

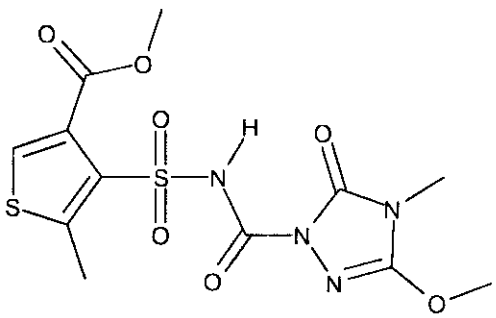
3. PRINCIPLE

The residues of BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide in water are determined by direct injection onto the LC/MS/MS system. Quantification is based on a comparison of peak areas with those of known standards.

The LOQ of the method is 0.5ng/mL(ppb) for BYH 18636 and its metabolites.

4. COMPOUNDS

Code Name: BYH 18636
(Parent Molecule)



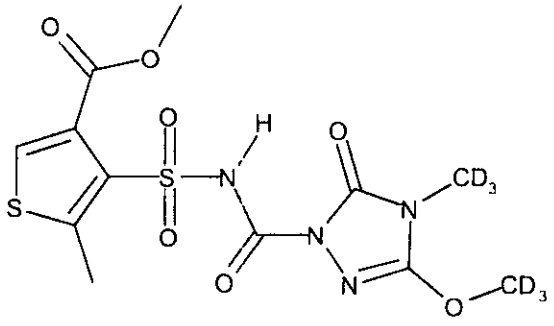
CAS Name: Methyl 4-[[[(4,5-dihydro-3-methoxy-4-methyl-5-oxo-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate

CAS Number 317815-83-1

Molecular Formula: $C_{12}H_{14}N_4O_7S_2$

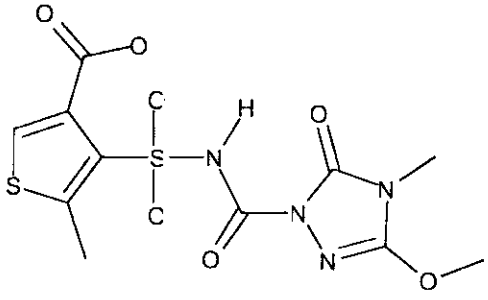
Molecular Weight: 390.3922

Code Name: BYH 18636-triazolinone-dimethyl-d_6
(Parent Molecule, Isotopic Internal Standard)



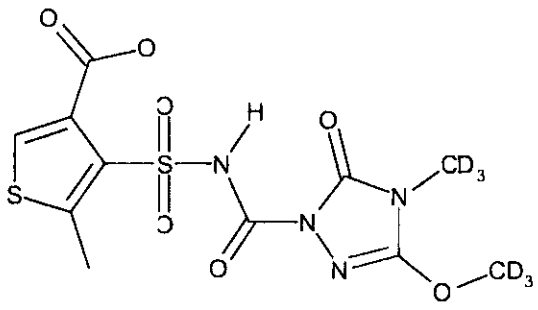
CAS Name: Methyl 4-[[[[4,5-dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylate
Molecular Formula: $C_{12}H_8D_6N_4O_7S_2$
Molecular Weight: 396.4291

Code Name: BYH 18636 Carboxylic acid
(Water Metabolite)



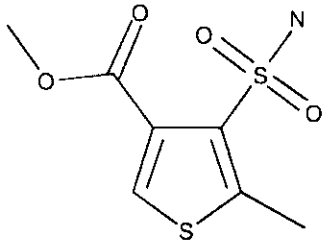
CAS Name: 4-[[[(4,5-Dihydro-3-methoxy-4-methyl-5-oxo-1H-1,2,4-triazol-1-yl)carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_{12}N_4O_7S_2$
Molecular Weight: 376.3656

Code Name: BYH 18636 Acid-triazolinone-dimethyl-d_6
(Water Metabolite, Isotopic Internal Standard)



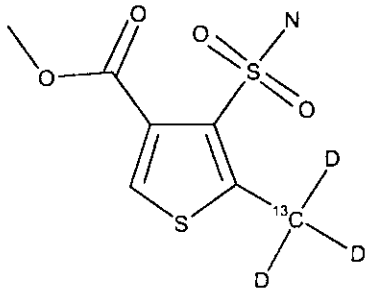
CAS Name: 4-[[[[4,5-Dihydro-3-(methoxy-d_3)-4-(methyl-d_3)-5-oxo-1H-1,2,4-triazol-1-yl]carbonyl]amino]sulfonyl]-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_{11}H_6D_6N_4O_7S_2$
Molecular Weight: 382.4025

Code Name: BYH 18636 Sulfonamide
(Water Metabolite)



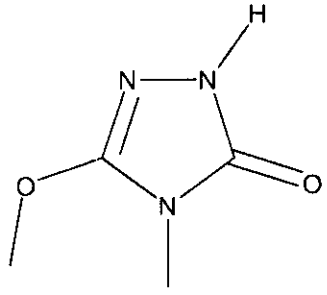
CAS Name: Methyl 4-(aminosulfonyl)-5-methyl-3-thiophenecarboxylate
CAS Number: 317815-81-9
Molecular Formula: $C_7H_9NO_4S_2$
Molecular Weight: 235 2807

Code Name: BYH 18636 Sulfonamide -5-methyl-$^{13}Cd_3$
(Water Metabolite, Isotopic Internal Standard)



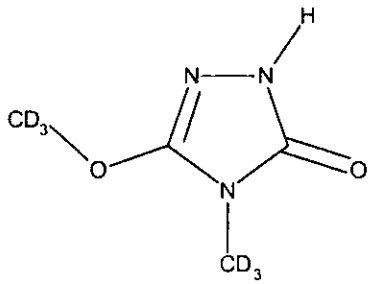
CAS Name: Methyl 4-(aminosulfonyl)-5-(methyl-^{13}C-d_3)-3-thiophenecarboxylate
Molecular Formula: $C_7H_6D_3NO_4S_2$
Molecular Weight: 239.3039

Code Name: Methoxytriazolinone
(Water Metabolite)



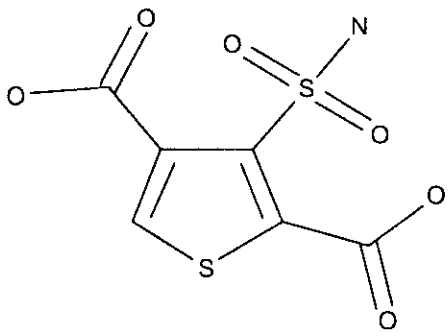
CAS Name: 2,4-Dihydro-5-methoxy-4-methyl-3H-1,2,4-triazol-3-one
CAS Number: 135302-13-5
Molecular Formula: $C_4H_7N_3O_2$
Molecular Weight: 129.1173

Code Name: Triazolinone-dimethyl-d_6
(Water Metabolite, Isotopic Internal Standard)



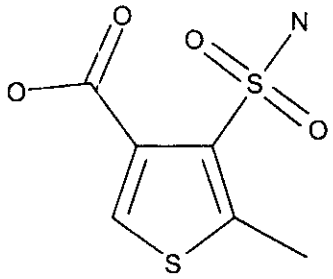
CAS Name: 2,4-Dihydro-5-(methoxy-d_3)-4-(methyl-d_3)-3H-1,2,4-triazol-3-one
Molecular Formula: $C_4HD_6N_3O_2$
Molecular Weight: 135.1542

Code Name: BYH 18636 Dicarboxy Sulfonamide
(Water Metabolite)



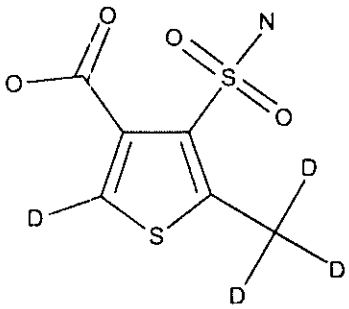
IUPAC Name: 3-(Aminosulfonyl)thiophene-2,4-dicarboxylic acid
Molecular Formula: $C_6H_5NO_6S_2$
Molecular Weight: 251.2370

Code Name: BYH 18636-sulfonamide carboxylic acid
(Water Metabolite)



CAS Name: 4-(Aminosulfonyl)-5-methyl-3-thiophenecarboxylic acid
Molecular Formula: $C_6H_7NO_4S_2$
Molecular Weight: 221.2541

Code Name: BYH 18636 Sulfonamide Carboxylic acid-2-d-methyl-d_3
(Water 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: 225.2787

5. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Eppendorf Reference Series 2000 pipettes (Cat. No.: 05-402-48 and 05-402-50)
- VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex® Brand disposable Pasteur pipets (Cat. No.: 53283-910 & 53283-914)
- National Scientific LC vials, Snap-Its (Cat. No.: C4011-5)
- National Scientific LC vial Snap-It Seals, (Cat. No.: C4011-55)
- Two LiChrospher® 60 RP-select B 5µ 125 x 3.00 mm Column (Part No.: 79925SB-563)
- Applied Biosystems PE Sciex 4000 LC/MS/MS System with Analyst Software Version 1.4.1 or higher installed
- Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10A VP Controller with a Perkin Elmer 200 Series autosampler
- VICI Cheminert Valve and 2 position actuator controller.
- Fisherbrand 125-mL glass jars (Cat. No. 02-911-455)

6. REAGENTS

Use as a guide; equivalents or different manufactures (brands) may be substituted.

- Acetonitrile, Fisher Scientifc Optima, , (Cat. No. A996-4)
- Deionized Water filtered through a Milli-Q water system or Water, Fisher Scientific Optima, (Cat. No.: W7-4)
- Acetic Acid, Guaranteed Reagent, (VRW Cat. No.: EM-AX0073-14)
- Formic Acid, 88% (J.T. Baker Cat No.0128-01)
- Certified analytical reference standards of BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide
- Certified internal standards of BYH 18636-triazolinone-dimethyl-d_6, BYH 18636 Acid-triazolinone-dimethyl-d_6, BYH 18636 sulfonamide acid-2-d-methyl-d_3, BYH 18636 sulfonamide-5-methyl-$^{13}Cd_3$ and BYH 18636-triazolinone-dimethyl-d_6.
- Solution of 10 ppm sodium thiosulfate: Weigh approximately 100 mg of sodium thiosulfate into a 100 mL volumetric flask. Dissolve the amount in approximately 50 mL of HPLC grade water and make up the volume to the 100 mL mark. Mix thoroughly by inverting the flask several times. This solution is 1mg/mL or 1000ppm. Transferring 1 mL of this solution to a 100 mL water sample will produce 10 ppm concentration of sodium thiosulfate in that sample. Transfer the sodium thiosulfate solution to 100 mL amber bottle and store refrigerated at ≤10°C.
- Solution of HPLC grade water chlorinated with sodium hypochlorite (NaOCl):
  Pipet 128 µL of NaOCl (13% chlorine, density 1.209 g/mL) into a 100 mL volumetric flask. Fill to volume with deionized, HPLC grade water. The resulting free chlorine concentration is 200 µg/mL. To simulate a chlorinated finished drinking water add an appropriate amount of this solution to a water sample. For example, add 100 µL of the 200 µg/mL free chlorine solution to a 10 mL HPLC grade water sample. The resulting level of free chlorine is 2µg/mL (ppm). Chlorine is volatile, so this solution should be

stored tightly sealed, in the dark under refrigeration at ≤10°C and should be remade if more than three weeks old.

7. PREPARATION OF ANALYTICAL STANDARDS

NOTE: The following procedure is an example description of how standard solutions may be prepared. Standards may be prepared as mixed solutions by dilution from individual stock solutions or prepared individually. Alternate or additional standards of appropriate weight and volume may be prepared as needed.

Class "A" volumetric glassware or calibrated pipets should be used in the preparation of all analytical standards. All standard solutions should be stored in a refrigerator in amber glass bottles when not in use. Solutions should be allowed to warm to room temperature prior to use.

7.1 Primary Stock Standard Solutions

Prepare individual 100 µg/mL stock solutions of BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide by transferring 0.0100 grams of each analyte in separate 100 mL volumetric flasks. Dilute to volume with acetonitrile and mix well.

Prepare a mixed stock 1.0µg/mL solution containing a mixture of BYH 18636 and its metabolites by taking a 1.0mL aliquot of each of the six 100 µg/mL stock solutions and diluting to 100 mL with acetonitrile.

NOTE: Corrections for standard purities should be applied when expressing standard concentrations.

7.2 Fortification Standard Solutions

Prepare a 0.1 µg/mL fortification solution containing a mixture of BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide by taking a 10.0mL aliquot of the 1 µg/mL standard solution and diluting to 100 mL with acetonitrile.

Further dilutions of this mixed fortification solution may be made as needed.

7.3 Isotopic Internal Standard Solutions

Note: As an internal standard for BYH 18636 dicarboxy sulfonamide is unavailable, triazolinone-dimethyl-d_6 is used as a surrogate internal standard.

Prepare individual 100µg/mL stock solutions of BYH 18636-triazolinone-dimethyl-d_6 BYH 18636 acid-triazolinone-dimethyl-d_6, BYH 18636 sulfonamide acid-2-d-methyl-d_3, BYH 18636 sulfonamide-5-methyl-$^{13}Cd_3$ and triazolinone-dimethyl-d_6 by transferring 0.005 grams of each analyte in separate 50 mL volumetric flasks. Dilute to volume with acetonitrile.

Prepare a 0.5µg/ml mixed stock internal standard solution of BYH 18636-triazolinone-dimethyl-d_6 and its metabolites BYH 18636 acid-triazolinone-dimethyl-d_6, BYH 18636 sulfonamide acid-2-d-methyl-d_3, BYH 18636 sulfonamide-5-methyl-13Cd_3, and BYH18636-triazolinone-dimethyl-d_6 by taking a 0.5mL aliquot of 100.0µg/mL stock solution and diluting to 100mL with acetonitrile

Further dilutions of this mixed fortification solution may be made as needed.

7.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.0, 0.1, 0.25, 0.5, 1, 5, and 10.0ng/mL of BYH 18636 and its metabolites diluted to 100mL with 10:90 v/v acetonitrile:deionized water and 0.4% formic acid. Before bringing the calibration solutions to volume, add by pipet 0.1mL of the 0.5µg/mL internal standard solution prepared in acetonitrile to each of the calibration solutions. (see Section 7.3 Isotopic Internal Standard Solutions)

Further calibration solutions may be prepared as needed.

Concentration of Standard Solution used for dilution (ng/mL)	Concentration of Internal Standard Solution used for dilution (ng/mL)	Aliquot Native mix Taken (mL)	Aliquot Internal Standard Taken (mL)	Dilution Volume (mL)	Concentration of Calibration Solution (ng/mL)
100	500	10.0	0.1	100	10.0
100	500	5.0	0.1	100	5.0
100	500	1.0	0.1	100	1.0
100	500	0.5	0.1	100	0.5
100	500	0.25	0.1	100	0.25
100	500	0.1	0.1	100	0.1
---	500	---	0.1	100	0.0

8. ANALYSIS OF FINISHED DRINKING WATERS (TAP WATERS) CONTAINING FREE CHLORINE

BYH 18636 and its metabolites degrade in water containing free chlorine. In order to accurately detect these residues when present in chlorine treated water, these residues would have to be stabilized at the time of sampling the water. Stabilization of residues for BYH 18636 and its metabolites can be achieved by adding sodium thiosulfate to the finished water sample at the time of collection. Sodium thiosulfate added to the water sample at 10 ppm concentration is sufficient to remove 2 ppm of chlorine and stabilize residues of BYH 18636 and its metabolites. For example, sample bottles that are used for collecting 100 mL samples of treated water should contain 1 mL of a 1000 ppm solution of sodium thiosulfate. Addition of the sodium thiosulfate to the sample bottles may be performed in the lab prior to transport to the water collection sites to prevent any potential contamination of the bottles in the field. The samples

so treated are then analyzed as per the method for non-free chlorine containing waters as described above.

Tap water or HPLC water chlorinated in the lab may be used for a finished drinking water method recovery sample. Appropriate amounts, for example, 100 µL of the 1000 ppm solution of sodium thiosulfate and 10mL of water, should be used, with the thiosulfate being added *before* the water is spiked with a known amount of a fortification solution to give the desired level of fortification. See Section 6 above for preparing free chlorine and thiosulfate solutions.

9. EXTRACTION

NOTE: This method uses internal standards to determine the concentrations of BYH 18636 and its metabolites present in water. If the concentrations of these components are outside the range of the appropriate calibration curve the analyses will have to be repeated using a reduced sample volume. If a further dilution is made to the final extract, adjust the concentration of internal standard added in step 9.3 so that the final concentration of internal standard present in the final sample is 0.5ppb.

1. Transfer 90 ± 2ml water sample to a 125ml glass jar. If the sample contains free chlorine, add sufficient sodium thiosulfate as described in Section 8.

2. Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile (see Section 7.2 Fortification Stock Solutions).

3. Add by pipet 0.1ml of the 0.5ppm internal standard solution prepared in acetonitrile. (see Section 7.3 Internal Standard Solutions).

4. Add 400µL of formic acid, and dilute to 100ml with acetonitrile. Cap and shake glass jar.

5. Filter the sample using an Acrodisc® 0.45µm syringe filter into a LC vial and cap to await analysis by LC/MS/MS.

10. ANALYSIS

10.1 Sample Analysis

BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide are analyzed by LC/MS/MS using isotopic internal standards.

Inject a 60 µL aliquot of each test sample (or fortified sample matrix) from step 5 in Section 9 onto the LC/MS/MS under the conditions presented in Appendix I. Variations in equipment or sample characteristics may require different injection volumes or slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity.

It is often beneficial to make several 'priming' injections of standards and/or samples prior to starting the LC/MS/MS analysis. Typically 4 to 6 priming injections are made. The results from these injections are not included in any calculations used in residue determinations. These injections help stabilize the LC/MS/MS response prior to running the analytical set.

10.2 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting an aliquot of each LC/MS/MS calibration solution interspersed with samples.

BYH 18636 and its metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide residues were quantified using internal standard linear regression analysis (As an internal standard for BYH 18636 dicarboxy sulfonamide is unavailable, triazolinone-dimethyl-d_6 is used as a surrogate internal standard). A separate calibration curve was produced for each set of samples analyzed on the LC/MS/MS. A calibration curve was generated by linear regression of the ratio of standard peak/internal standard peak areas versus the standard concentrations in ng/mL using Applied Biosystems Analyst Software (Version 1.4.1), a computer-programmed data capturing system. The Analyst Software uses the MS/MS standard responses to calculate the regression coefficients M and B, respectively called slope and intercept, for each analytical set.

The standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

The equation shown below is for the calculation of BYH 18636 residues.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of BYH 18636 in water was calculated using the following equation,

$$\text{BYH 18636 found (ppb)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{100}{90} = 1.11$$

Analyst software was used to calculate the amount of BYH 18636 in ppb for each sample and the percent recovery for the fortified samples.

10.3 Fortification Experiments

Note: Fortification experiments may be performed as needed to monitor method efficiency and reproducibility, but are not required when analysis of samples is performed for tolerance enforcement. Fortification experiments are intended to

be used for data collection methods or establishing & validating method efficiency.

With each sample set, analyze an untreated control sample and one or more fortified control samples. Calculate recoveries using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control sample, real or apparent
T = theoretical ppb in fortified sample

Recoveries are determined by analyzing fortified control samples alone or in conjunction with a sample set. Samples may be fortified prior to extraction at the LOQ of 0.5ppb in water or other appropriate level with fortification solutions. Calculate the final residue R for the control (S) and fortified control (R) samples.

11. DISCUSSION

11.1 Method Validation

The method validation has been performed and reported in Bayer CropScience Study RAGSM001[1]. The results from this study are summarized in Appendix 3.

11.2 Independent Laboratory Validation (ILV)

An ILV has been successfully performed on a prior version of the method[2]. The method was updated to incorporate the results and findings of the ILV and assigned a method number of GS-004-W06-02. The validation results are summarized in Table 2 of this report.

11.3 Confirmatory Method

The analytical method employs highly specific and selective detectors (LC/MS/MS), therefore it was not deemed necessary to develop a confirmatory method. However, if unexpected interferences are detected alternate ion transitions may be monitored. The following alternate ions are suggested, and the spectra for each of the analytes are presented in Appendix 4.

Analyte	Primary Ion Transition			Alternate Ion Transition		
	Polarity	Q1	Q3	Polarity	Q1	Q3
BYH 18636	+	391	359	+	391	230
BYH 18636-carboxylic acid	-	375	202	+	377	359
BYH 18636-sulfonamide	+	236	204	+	236	219
BYH 18636-sulfonamide carboxylic acid	-	220	176	-	220	80
BYH 18636-MMT	+	130	115	+	130	58
BYH 18636-dicarboxy sulfonamide	-	250	162	-	250	206

11.4 Time Considerations

A set of fourteen samples can be prepared for analysis in 2-3 hours, analyzed overnight and the data processed the following working day.

12. REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1	RAGSM001		**Wade, J.M.**, In House Laboratory Validation of the Analytical Method for the Determination of Residues of BYH 18636 And Its Metabolites BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide In Water Using LC/MS/MS
2	MR-07/218	P 614 067075	**Krebber, R., and Leppelt, L.**, Independent Laboratory Validation Of Method Gs-004-W06-01 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

Table 1 Analytical Method Summary Parameters (DER Table B.1.1)

Summary Parameters for the Analytical Method Used for the Quantitation of BYH 18636, BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide Residues in Water.	
Method ID	GS-004-W06-02
Analyte(s)	BYH 18636, BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide
Extraction solvent / Technique	Direct inject
Cleanup Strategies	none
Instrument Detector Column	- Two Shimadzu LC-10AD VP HPLC pumps with a Shimadzu SCL-10 controller and Perkin Elmer 200 Series autosampler - Applied Biosystems API 4000 MS/MS - 2 LiChrospher® 60 RP-select B 5 µm 125 x 3.0m coupled.
Standardization Method	Multi point calibration curve (Internal standard)
Stability of Standard Solutions	Stock standard solutions are stable for a minimum of 3 months when stored in the dark at =-18°C Fortification and calibration standard solutions are stable for a minimum of 1 month when stored in the dark at =4°C
Retention times	BYH 18636-dicarboxy sulfonamide(~4.6 minutes) BYH 18636-MMT (~6.1 minutes) BYH 18636-sulfonamide carboxylic acid(~8. 5 minutes) BYH 18636-sulfonamide(~10.5 minutes) BYH 18636-carboxylic acid(~10.8 minutes) BYH 18636(~12.5 minutes)

Appendix 1 Instrument Conditions For BYH 18636 and its metabolites

Equipment with equivalent or better sensitivity and performance may be substituted.

LC/MS/MS Parameters

NOTE: As the LC/MS/MS system is used over time, system components slowly and gradually become contaminated which in turn decreases system performance. The chromatographic response and/or peak shape of one or more of the analytical targets may be gradually affected over time. Therefore, the given LC/MS/MS parameters listed below are guidelines of where to start. Each instrument has its own unique personality. Variations in equipment or sample characteristics may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. These parameters should be optimized for the instrument and column actually used. Instrument parameters and mobile phase may be adjusted to improve separation from interfering peaks.

Acquisition Parameters

Instrument Used:	Perkin Elmer Sciex API 4000 LC/MS/MS System with Valco Divert Valve
Interface:	PE Sciex Turbo Ion Spray Electrospray
Synchronization Mode:	LC Sync
AutoEquilibration:	Off
Acquisition Duration:	21 min. 43 sec.
Periods in File:	5
Acquisition Module:	Acquisition Method
Software Version:	Analyst 1.4.1

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Low
Intensity Thres.:	0.00 cps
Settling Time:	700.0000 msec
MR Pause:	5.0070 msec
MCA:	No

Appendix I (continued)

Analyte (~4.7 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-Dicarboxy Sulfonamide	250	162	600	DP	-30	-30
				EP	-15	-15
				CE	-16	-16
				CXP	-5	-5

Parameter Table		
	CUR:	15
	GS1:	70
	GS2:	70
	IS:	-4200
	TEM:	500
	CAD:	6

Period 2 Experiment 1:		
	Scan Type:	MRM (MRM)
	Polarity:	Positive
	Scan Mode:	N/A
	Ion Source:	Turbo Spray
	Resolution Q1:	Low
	Resolution Q3:	Low
	Intensity Thres.:	0.00 cps
	Settling Time:	700.0000 msec
	MR Pause:	5.0070 msec
	MCA:	No
	Step Size:	0.00 amu
	Scan Type:	MRM (MRM)

Analyte (~6.15 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-MMT	130	115	500	DP	61	61
				EP	10	10
				CE	25	25
				CXP	8	8

Appendix I (continued)

Analyte (~6.15 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-MMT Internal Standard	136	118	500	DP	61	61
				EP	10	10
				CE	25	25
				CXP	8	8

Parameter Table

CUR:	15
GS1:	70
GS2:	70
IS:	4200
TEM:	500
CAD:	6

Period 3 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Low
Resolution Q3:	Low
Intensity Threshold:	0.00 cps
Settling Time:	700.0000 msec
MR Pause:	5.0070 msec
MCA:	No
Step Size:	0.00 amu

Analyte (~8.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Parameter	Start	Stop
BYH 18636- Acid Sulfonamide	220	176	500	DP	-30	-30
				EP	-10	-10
				CE	-16	-16
				CXP	-15	-15

Analyte (~8.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Parameter	Start	Stop
BYH 18636- Acid Sulfonamide Internal Standard	224	180	500	DP	-30	-30
				EP	-10	-10
				CE	-16	-16
				CXP	-15	-15

Appendix I (continued)

Parameter Table

CUR:	10
GS1:	70
GS2:	70
IS:	-4200
TEM:	500
CAD:	6

Period 4 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Low
Resolution Q3:	Low
Intensity Threshold:	0.00 cps
Settling Time:	700.0000 msec
MR Pause:	5.0070 msec
MCA:	No
Step Size:	0.00 amu

Analyte (~10.9 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-Carboxylic Acid	375	202	300	DP	-40	-40
				EP	-10	-10
				CE	-18	-18
				CXP	-15	-15

Analyte (~10.9 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-Carboxylic Acid Internal Standard	381	202	300	DP	-40	-40
				EP	-10	-10
				CE	-18	-18
				CXP	-15	-15

Appendix I (continued)

Period 4 Experiment 2:

Scan Type:	MRM (MRM)
Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Threshold:	0.00 cps
Smart Settling:	700.0000 msec
Settling Time:	5.0070 msec
MR Pause:	No
MCA:	0.00 amu
Step Size:	MRM (MRM)

Analyte (~10.8 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-Sulfonamide	236	204	300	DP	36	36
				EP	10	10
				CE	15	15
				CXP	14	14

Analyte (~10.8 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636-Sulfonamide Internal Standard	240	208	300	DP	36	36
				EP	10	10
				CE	15	15
				CXP	14	14

Parameter Table

CUR:	10
GS1:	70
GS2:	70
IS:	4200
TEM:	500
CAD:	6

Appendix I (continued)

Period 5 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Scan Mode:	N/A
Ion Source:	Turbo Spray
Resolution Q1:	Low
Resolution Q3:	Low
Intensity Threshold:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0070 msec
MCA:	No
Step Size:	0.00 amu

Analyte (~12.5 Min.)	Q1 Mass (amu)	Q3 Mass(amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636	391	359	500	DP	30	30
				EP	10	10
				CE	14	14
				CXP	14	14

Analyte (~12.5 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
BYH 18636 Internal Standard	397	365	500	DP	30	30
				EP	10	10
				CE	14	14
				CXP	14	14

Parameter Table		
	CUR:	10
	GS1:	70
	GS2:	70
	IS:	4200
	TEM:	500
	CAD:	7

Appendix I (continued)

PE200 Autosampler Properties

Inject Details	
Syringe Size (µl):	250
Injection Volume (µl):	60
Flush Details	
Pre-inject Flushes (#):	1
Post-inject Flushes (#):	9
Inject Details (Advanced)	
Air Cushion (µl):	10
Excess Volume (µl):	10
Sample Speed:	Fast
Needle Level (%):	10
Inject Delay Time (min):	0.00
Replicate Injections (#):	1
Analysis Time (min):	0.00
Vial Vent Mode:	On
Loop Mode:	Partial
Loop Volume (µl):	200
Flush Details (Advanced)	
Flush Volume (µl):	700
Flush Speed:	Medium
Temperature Control:	Enable

HPLC Parameters

Pumps Used:	Two Shimadzu LC-10ADVP (High Pressure Mixer) pumps with a Shimadzu SCL-10 controller	
Minimum Pressure:	0.0 psi	
Maximum Pressure:	4000 psi	
Shutdown Time:	999.9 min.	
Column Temperature:	Ambient	
Column:	Manufacturer:	LiChrospher®
	Type:	60 RP-select B
	Particle Size:	5 µm
	Diameter:	3.0 mm
	Length:	125 mm
Note:	This method requires two LiChrosphere® 60RP-select B columns to be coupled.	
	Mobile Phase A:	0.5% Formic Acid in Water (v/v)
	Mobile Phase B:	0.1% Formic Acid in Acetonitrile (v/v)

Appendix I (continued)

Gradient Program:

Step	Time (min.)	Module	Flow Rate (mL/min)	A(%)	B(%)
0	0.10	Pumps	0.30	90.0	10.0
1	3.00	Pumps	0.30	80.0	20.0
2	9.00	Pumps	0.30	60.0	40.0
3	10.50	Pumps	0.60		
4	11.50	Pumps	0.60	10.0	90.0
5	15.45	Pumps	0.30		
6	15.50	Pumps	0.30	10.0	90.0
7	15.51	Pumps	0.30	90.0	10.0
8	20.00	Pumps	0.30	90.0	10.0
9	20.10	System Controller	Stop		

Valco Divert Valve Program:

Step	Total Time (min.)	Divert Location
1	0.0	To Waste
2	3.5	To LC/MS
3	14.5	To Waste

Appendix 2 Example Calculation

An example calculation for BYH 18636 from sample RAGSM001-Surface Water-LOQ-2, which was analyzed during the method validation study is presented below. This sample was fortified with 0.5ppb BYH 18636, BYH 18636-carboxylic acid, BYH 18636-sulfonamide, BYH 18636-sulfonamide carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide. The chromatogram used in this example is presented in Appendix 4 (Chromatogram 5) and the calibration curve for this analysis is presented in Appendix 5.

The standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

The example shown below is for the calculation of BYH 18636 residues. BYH 18636-sulfonamide-carboxylic acid, BYH 18636-MMT, and BYH 18636-dicarboxy sulfonamide residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The parts per billion (ppb) of BYH 18636 in water was calculated using the following equation,

$$\text{BYH 18636 found (ppb)} = \frac{(Y-B) \times D}{M}$$

$$\text{Where Dilution Factor (D)} = \frac{\text{Final dilution volume } (V_2)}{\text{Initial volume}(V_1)}$$

V_1	V_2	Native Peak Area	IS Peak Area	Y	M	B
90mL	100mL	165722.5	171573.7	0.9659	2.039	0.0184

From the above equations:

$$\text{Dilution Factor (D)} = \frac{100}{90} = 1.11$$

$$\text{BYH 18636 found} = \frac{(0.9659-0.0184) \times 1.11}{2.039} = 0.5158 \text{ ppb}$$

Therefore sample RAGSM001-Surface Water-LOQ-2 contains 0.5158ppb BYH 18636.

The % recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{(R - S)}{T} \times 100$$

Where: R = ppb of target analyte found in fortified sample
S = ppb of target analyte found in control samples, real or apparent
T = theoretical ppb in fortified sample

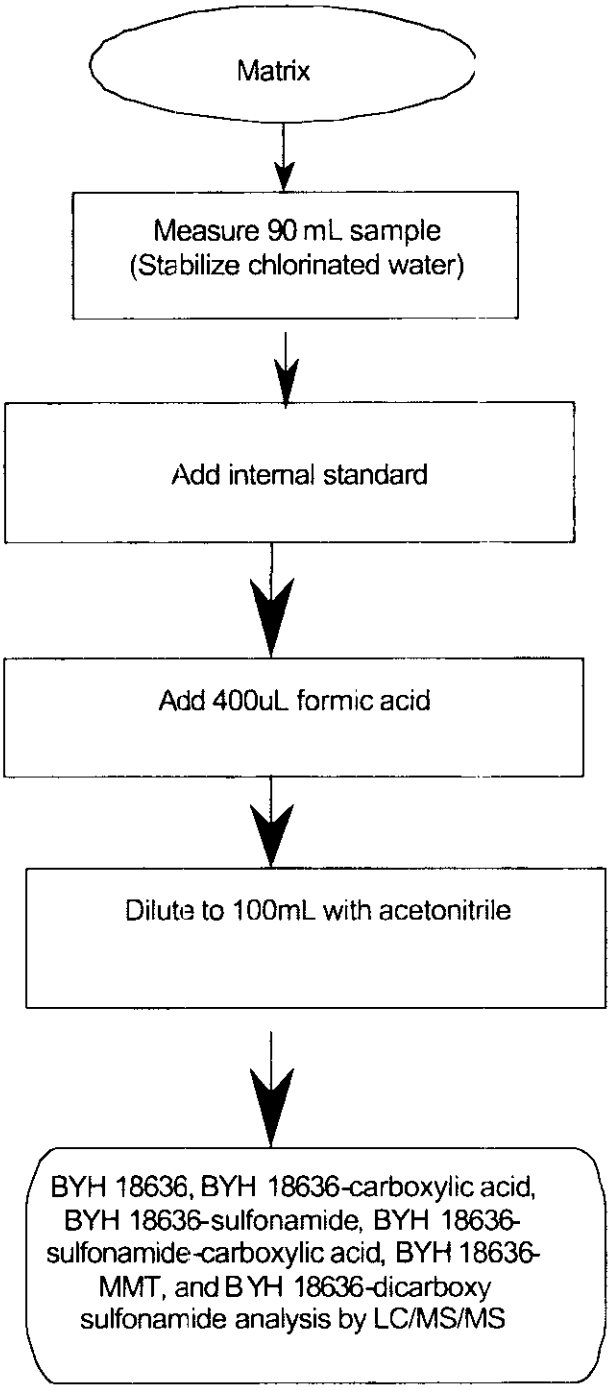
Therefore, for sample RAGSM001-Surface Water-LOQ-2, which was fortified with 0.5ppb BYH 18636:

R = 0.5158 ppb
S = 0.00079ppb
T = 0.5 ppb

$$\text{\% BYH 18636 Recovery} = \frac{(0.5158 - 0.00079)}{0.5} \times 100 = 103\%$$

Note: The above calculations were performed using rounded numbers and may vary slightly from the results presented in the raw data

Appendix 6 Method Flow Chart



Appendix 7 Revision History

Method #	Revision	Description
GS-004-W06-01	01	Method prepared on completion of validation study[1]
GS-004-W06-02	02	Method prepared on completion of ILV study[2]