2. BACKGROUND INFORMATION

The objective of this study was to validate method of analysis for the determination of AE 0317309 and Its Metabolite AE B197555 in Water by LC/MS/MS, which was described in the study protocol.

On completion of this study the analytical method AI-003-W05-01: "AE 0317309: Analytical Method For The Determination of AE 0317309 And Its Metabolite AE B197555 In Water by LC/MS/MS" was prepared.

The study was performed in accordance with United States Environmental Protection Agency (EPA) Pesticide Assessment Guidelines and Good Laboratory Practices (Residue Chemistry Test Guidelines, OPPTS 860.1340[1] and 850.7100[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 AE 0317309 and AE B197555 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 May 26, 2005. The experimental phase of the study began on May 27, 2005 and concluded on August 29, 2005.

The following personnel were involved in the conduct of this study:

Derek J. Netzband
Senior Scientist
Environmental Research
17745 South Metcalf Avenue
Stilwell, Kansas 66085

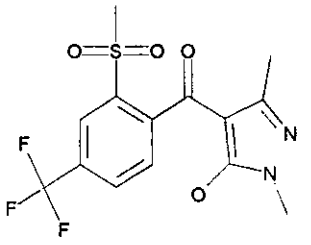
Jessica Yin
Research Scientist
Environmental Research
17745 South Metcalf Avenue
Stilwell, Kansas 66085

Jami Wade
Research Associate
Environmental Research
17745 South Metcalf Avenue
Stilwell, Kansas 66085

4. TEST AND REFERENCE SUBSTANCES

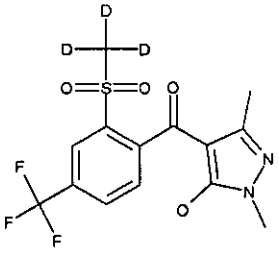
The following compounds were used as test and reference substances, and were supplied by Bayer CropScience. Neat standards were stored in a freezer at or below approximately –10°C. Standard solutions were stored in a refrigerator at approximately 4°C.

Code Name: AE 0317309
(Active Ingredient, Parent Molecule)



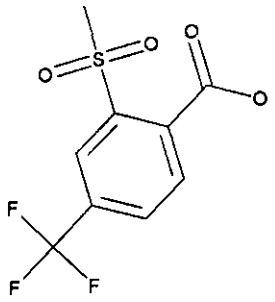
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone
CAS Number [365400-11-9]
Molecular Formula: $C_{14}H_{13}F_3N_2O_4S$
Molecular Weight: 362.33
ID No.: K-1394
Reference No.: 0324200303
Purity: 99.5%
Expiration Date: 26 October 2008
Storage Conditions: Frozen

Code Name: AE 0317309-*methylsulfonyl*-d_3
(Parent Molecule, Deuterated Internal Standard)



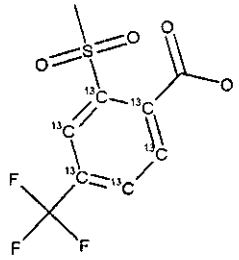
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone-methylsulfonyl-d_3
Molecular Formula: $C_{14}H_{10}D_3F_3N_2O_4S$
Molecular Weight: 365.35
ID No.: K-1261
Reference No.: 2001BRP113-37
Purity: 96.1%
Expiration Date: 11 August 2013
Storage Conditions: Frozen

Code Name: AE B197555 (or RPA2303328)
(Metabolite)



CAS Name: 2-(Methylsulfonyl)-4-(trifluoromethyl)benzoic acid
CAS Number: [142994-06-7]
Molecular Formula: $C_9S0_4F_3H_7$
Molecular Weight: 268.21
ID No.: K-1367
Reference No.: IGB947
Purity: 99.6%
Expiration Date: 27 September 2009
Storage Conditions: Frozen

Code Name: AE B197555-*phenyl* $^{13}C_6$ (or AE1345453-*phenyl* $^{13}C_6$)
(Metabolite, ^{13}C Internal Standard)



CAS Name: 2-(Methylsulfonyl)-4-(trifluoromethyl)benzoic acid-phenyl $^{13}C_6$
Molecular Formula: $C_3{}^{13}C_6S0_4F_3H_7$
Molecular Weight: 274.14
ID No.: K-1217
Reference No.: GAR1892/5
Purity: 96.7%
Expiration Date: 28 January 2013
Storage Conditions: Frozen

5. TEST SYSTEM – WATER SAMPLES

The method was validated using one surface water, one ground water and one tap water. The surface control water used in this study was collected for Bayer CropScience Study Number 00M19458, Ethoprophos Surface Water Monitoring. The ground control water used in this study was collected for Bayer CropScience Study Number MEGUY003, Survey of Azinphos-Methyl (GUTHION) Residues in Well Water taken From Agricultural Areas of Virginia , Pennsylvania, and New York. The treated tap control water was collected from a treated drinking water tap at Bayer Research Park, Stilwell, Kansas.

The table below gives the sample ID, water type and sampling location for each of the waters. The raw data regarding the water sample identity and characterization are archived with the original studies listed below:

Sample ID	Original Study	Water Type	Source Location
19458-CA0097	00M19458[4]	surface water	Lodi, CA
MK-2-9/10/04-A	MEGUY003[3]	ground water	Biglerville, PA
BRP002	MEAIX018	treated tap water	Stilwell, KS

6. STORAGE

The untreated ground water samples were stored frozen at about - 10°C and the surface waters in a refrigerator at approximately 4°C. The treated tap water was taken fresh for processing and was not stored before or during the study.

7. REAGENTS AND EQUIPMENT

7.1 Reagents and General Equipment

The apparatus and reagents used in this study are listed in Sections 4 and 5 of the method of analysis presented in Appendix 2.

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 AE 0317309 and its metabolite AE B197555 in water were determined using two separate LC/MS systems. An Applied Biosystems / Sciex API-3000 LC/MS/MS system, using Analyst software (Version 1.2), with Sciex Turbolon Spray Electrospray Interface; Shimadzu LC-10AD VP HPLC pumps (2) with a high pressure mixer and SCL-10A VP Pump Controller; and a Perkin Elmer 200 Series Autosampler was used for surface and tap water analyses. An Applied Biosystems / Sciex API-4000 LC/MS/MS system, using Analyst software (Version 1.4.1), with Sciex TurbolonSpray Electrospray Interface; Shimadzu LC-10AD VP HPLC pumps (2) with a high

pressure mixer and SCL-10A VP Pump Controller; and a Gilson 215 Series Autosampler was used for ground water analyses. The operating parameters used are outlined in Appendix I of the analytical method, which is presented in Appendix 2 of this report.

8. CALCULATIONS

8.1 Calibration Curves

At least six different standard concentrations were run with each set of samples.

Standard concentrations of AE 0317309 and AE B197555 ranged from 0.0 ng/mL to 20.0 ng/mL (ppb), each with 2.0 ng/mL isotopic internal standard added. The calibration standards were interspersed with the samples. All calculations were performed using Applied Biosystems Analyst software 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

AE 0317309 and AE B197555 residues were quantified using internal standard linear regression analysis. 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, 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[a]
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area: isotopic peak area ratio

[a] As the concentration of the internal standard is the same in both the calibration standards and fortified samples, it may be omitted from these calculations.

The example shown below is for the calculation of AE 0317309 residues. AE B197555 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 AE 0317309 in water was calculated using the following equation,

$$\text{AE 0317309 found (ppb)} = \frac{(Y-B) \times D}{M}$$

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

Analyst software was used to calculate the amount of AE 0317309 in ppb for each sample and the percent recovery for the spiked samples.

An example calculation from the validation of AE 0317309 in tap water is described below.

An example calculation for AE 0317309 from sample Tap Water LOQ 3 obtained during the validation of AE 0317309 and AE B197555 in tap water is shown below.

V_1	V_2	Native Peak Area	IS Peak Area	Y	M	B
100	5	42771.9	97689.3	0.4378	0.4951	0.0524

From the above equations:

$$\text{Dilution Factor (D)} = \frac{5}{100} = 0.05$$

$$\text{AE 0317309 found} = \frac{(0.4378-0.0524) \times 0.05}{0.4951} = 0.0389 \text{ ppb}$$

Therefore 0.0389ppb of AE 0317309 was detected in sample Tap Water LOQ 1.

The % Recovery in each sample set was calculated using the following formula:

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

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

In the above example, 0.0389ppb of AE 0317309 was detected in the fortified control sample, and no apparent residue was detected in the control sample. As the sample was initially fortified with 0.05ppb AE 0317309 the % recovery was calculated using the following equation:

$$\% \text{ AE 0317309 Recovery} = \frac{(0.0389 - 0.00)}{0.05} \times 100 = 78\%$$

A Microsoft Excel spreadsheet summarizing the AE 0317309 data collected during the method validation in tap water is presented in Figure 1. The control sample, fortification sample and representative standards from this validation set are presented in Appendix 1.

Note: These example calculations were performed using rounded numbers, and therefore may be slightly different from the value shown in the raw data.

12. REFERENCES

No.	Doc. No.	Report No.	**Author**, Title. Year.
1			U.S. EPA Ecological Effects Test Guidelines OPPTS 850.7100 Data Reporting for Environmental Research Methods, Public Draft, April 1996.
2			U.S. EPA Residue Chemistry Test Guidelines OPPTS 860.1340 Residue Analytical Method, August 1996.
3		MEGUY003	Survey of Azinphos-Methyl (GUTHION) Residues in Well Water taken From Agricultural Areas of Virginia , Pennsylvania, and New York, 2004.
4		00M19458	**J. B. Van Kretschmar and S. Daussin,** Surface Water Monitoring for Residues of Ethoprop in High-Use Areas in the United States, Progress Report, July 25, 2002.
5	P 614 047066	MR-139/05	**R. Krebber,** Independent Laboratory Validation Of Method AI-003-W05-01 For The Determination Of AE 0317309 And Its Metabolite AE B197555 In Water, 2005
6		AI-003-W05-02	**D.J. Netzband**, AE 0317309: Analytical Method For The Determination Of AE 0317309 And Its Metabolite AE B197555 In Water by LC/MS/MS

Appendix 2: Analytical Method AI-003-W05-02

AE 0317309: Analytical Method For The Determination of AE 0317309 And Its Metabolite AE B197555 In Water by LC/MS/MS

2. BACKGROUND

The herbicide AE 0317309 is currently being developed by Bayer CropScience. AE 0317309 has potential uses in several crops

An analytical method was developed for the analysis of AE 0317309 and its associated metabolite AE B197555 in water, and the method was validated in Bayer CropScience Study Number MEAIX018[1]. The structures for these compounds are presented in Appendix 2. 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 were obtained from the method validation study.

3. PRINCIPLE

AE 0317309 and AE B197555 are extracted from the water using solid phase extraction (SPE) procedure. After elution from SPE column, the compounds are analyzed by LC/MS/MS. The LC/MS/MS technique allows quantitation of all analytical targets with a high inherent specificity and without the need of derivitization for the more polar analytes.

An aliquot of water is fortified with an isotopic internal standard containing AE 0317309-d_3 and AE B197555-$^{13}C_6$, acidified, cleaned up using a RP-102 SPE cartridge, and diluted to 5mL in 90:10 0.1% acetic acid in water/acetonitrile to await analysis by LC/MS/MS for AE 0317309 and AE B197555.

A *flow-chart outlining the procedure summarizes the method in Appendix 6.*

Additional summaries outlining the method parameters and method characteristics are presented in Table 1 and Table 2.

4. APPARATUS

Use as a guide; equivalent apparatus may be substituted.

- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Rainin Microman® Classic positive-displacement pipettes (Cat. No.: M-25, M-50, and M-250)
- VWR Pyrex® Brand graduated cylinder (Cat. No.: 24760-100)
- 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)
- Acrodisc ® CR13mm syringe filter with 0.45µm PTFE membrane (Catalog No 4422T)
- Javelin Direct-Connect Column Filter, 2.1 mm, (Part No.: 88200)
- Phenomenex Prodigy 5µ C8 50 x 2.00 mm Column (Part No.: 00B-3301-B0)
- Applied Biosystems PE Sciex 3000 LC/MS/MS System with a Dell Optiplex PC and Analyst Software Version 1.2 or higher installed.
- Applied Biosystems Turbo Ionspray Interface.

- Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10AVP Controller with a Gilson 215 Liquid handler and Gilson 819 Valve Actuator.
- VICI Cheminert Valve and 2 position actuator Controller.
- Applied Separations 200mg/3mL RP-102 Resin Spe-ed SPE Cartridges (Catalog No 4208)
- 60mL Solvent reservoir (Varian Catalog Number 12131012)
- Analytical Balance with accuracy of ± 0.01 grams for sample weights and ± 0.0001 grams for analytical standards.

5. REAGENTS

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

- Acetonitrile, EM Science Omnisolv, (VWR Cat. No.: EM-AX0145-1)
- Deionized Water filtered through a Milli-Q water system or:
- Water, EM Science Omnisolv, (VWR Cat. No.: EM-WX0004-1)
- Acetic Acid, Guaranteed Reagent, (VRW Cat. No.: EM-AX0073-14)
- Formic Acid, 88% (J.T. Baker Cat No.0128-01)
- Sodium hypochlorite (13% free chlorine), Acros Organics
- Sodium thiosulfate (100% purity), Mallinckrodt
- Certified analytical reference standards of AE 0317309 and AE B197555.
- 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 2 µg/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.

6. 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 amber glass bottles

at or below 10°C when not in use. Solutions should be allowed to warm to room temperature prior to use.

6.1 Primary Stock Standard Solutions

Prepare individual 200 µg/mL stock solutions of AE 0317309 and AE B197555 by placing 0.0100 grams of each analyte in separate 50 mL volumetric flasks. Dilute to volume with acetonitrile.

NOTE: Corrections for standard purities should be applied when expressing standard concentrations. For Example: If an analytical standard material has a purity of 98.0%, then 0.0102 grams (0.0100 g / 0.980) would be required to prepare a 200 µg/mL stock solution.

The stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤-18°C.

6.2 Fortification Standard Solutions

Prepare a stock 10 µg/mL solution containing a mixture of AE 0317309, AE B197555 by taking a 5 mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.
Prepare a 0.05 µg/mL fortification solution containing a mixture of AE 0317309 and AE B197555 by taking a 0.5 mL aliquot of the 10 µg/mL stock solution and diluting to 100 mL with acetonitrile.
Prepare a 0.005 µg/mL fortification solution containing a mixture of AE 0317309 and AE B197555 by taking a 10 mL aliquot of the 0.05 µg/mL stock solution and diluting to 100 mL with acetonitrile.
Further dilutions of this mixed fortification solution may be made as needed.

The fortification standard solutions are stable for a minimum of 3 months when stored in the dark at ≤4°C.

6.3 Isotopic Internal Standard Solutions

Prepare individual 100 µg/ml stock internal standard solutions of AE 0317309-d_3 and AE B197555-$^{13}C_6$ by placing 0.0050 grams of each analyte in separate 50 mL volumetric flasks. Dilute to volume with acetonitrile.
Prepare a stock 10µg/mL solution containing a mixture of AE 0317309-d_3 and AE B197555-$^{13}C_6$ by taking a 10.0 mL aliquot of each stock solution and diluting to 100 mL with acetonitrile.
Prepare a 0.1µg/mL solution containing a mixture of AE 0317309-d_3 and AE B197555-$^{13}C_6$ by taking a 1.0 mL aliquot of the 10µg/mL AE 0317309-d_3 and AE B197555-$^{13}C_6$ and diluting to 100 mL with 10% acetonitrile/90% 0.1%Acetic Acid in deionized water.

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

6.4 Calibration Standard Solutions

Prepare working calibration solutions consisting of 0.0, 0.2, 0.5, 2.0, 5.0, 10.0 and 20.0ng/mL of AE 0317309 and AE B19755 diluted to 100mL with 10:90 acetonitrile: 0.1% (v/v) acetic acid in deionized water Before bringing the calibration solutions to volume, add by pipet 2.0mL of the 0.1µg/mL internal standard solution prepared in 10% acetonitrile/90% 0.1% acetic acid in deionized Water to each of the calibration solutions. (see Section 6.3 Internal Standard Solutions)

Further calibration solutions may be prepared as needed.

7. ANALYTICAL PROCEDURE FOR ANALYSIS OF WATER

A method flow chart is presented in Appendix 6, and a summary of the analytical method parameters is presented in Table 1.

Stopping points in the analytical method are designated by the following symbol: ♦

7.1 Analysis of Finished Drinking Waters (Tap Waters) Containing Free Chlorine

AE 0317309 degrades in water containing free chlorine (see Table 6) to AE B197555. 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 AE 0317309 residues 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 AE 0317309 residues. 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 done 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 5 for preparing free chlorine and sodium thiosulfate solutions.

7.2 Sample Preparation

Samples of water should be stored frozen until sampled for extraction. Samples are brought to room temperature. Mix the sample completely before removing a sub-sample for analysis

7.3 Analysis of Water Samples by LC/MS/MS

Note: Fortification experiments may be used for establishing and validating method efficiency as required.

7.3.1 Transfer 100± 2mL of water to a stoppered measuring cylinder. ♦

7.3.2 Fortify the recovery samples at the desired fortification level with the appropriate mixed standard solution prepared in acetonitrile (see Section 6.2 Fortification Stock Solutions).

7.3.3 Add by pipet 100µL of the 0.1µg/mL internal standard solution prepared in 10% acetonitrile/90% 0.1% acetic acid in DI Water. (see Section 6.3 Internal Standard Solutions). Add 6mL of formic acid to the contents of each measuring cylinder. Stopper the cylinder and mix the contents.

7.3.4 Set-up a RP-102 cartridge (200 mg) on a purification system. Piggy back a solvent reservoir on top of the cartridge. Condition the cartridge with one column volume (~2.5mL) of 50:50 acetonitrile/methanol followed by one column volume of HPLC water. (~2 mL/min. Do not allow the cartridge to dry).

7.3.5 Transfer the contents of the measuring cylinder to the cartridge. Elute and discard the effluent (flow rate ~2 drop/ sec).

7.3.6 Add ~1.0 mL of a solution of 0.8 % formic acid in water to the cartridge. (~1 drop/2 sec. Do not allow the cartridge to dry). Elute and discard the effluent.

7.3.7 Add ~ 1.0 mL of water to the cartridge (~1 drop/2 sec. Do not allow the cartridge to dry). Elute and discard the effluent.

7.3.8 Dry the cartridge for ~2 minutes. Vacuum or positive nitrogen pressure may be used to dry the cartridge. If the samples are prepared on a vacuum manifold system, then ~20 inches of mercury vacuum could be used

7.3.9 Add ~1.0 mL of 50:50 acetonitrile/methanol to the cartridge. Apply vacuum and push the solvent onto the cartridge. Take precautions to insure that no eluent is lost. Vent the vacuum and allow the cartridge to soak for 1-2 minutes. Reapply vacuum and elute all solvent (~1drop/second) into a 10mL test tube with a screw top lid.

7.3.10 Add ~4mL 0.1% (v/v) acetic acid in deionized water. Stopper and mix the sample well. Filter the sample using an Acrodisc® 0.45µm syringe filter into a LC vial to await analysis by LC/MS/MS. ♦ (Refrigerate the extract at ~4 °C if not analyzed immediately).

8. ANALYSIS

8.1 Sample Analysis

AE 0317309 and AE B197555 are analyzed by LC/MS/MS using isotopic internal standards.

Inject a 40 µL aliquot of each test sample (or fortified sample matrix) from step 7.3.10 into 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.

8.2 Standard Calibration

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting a 40 µL aliquot of each LC/MS/MS calibration solution interspersed with samples. Construct a standard curve for the analytes of interest by plotting the ratio of the native peak count/internal standard count vs. the standard concentration. Obtain the least square regression line of this data.

The equations used and example calculations are presented in Appendix 4.

8.3 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:

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

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 0.05ppb in

water or other appropriate level with fortification solutions. Calculate the final residue R for the control (S) and fortified control (R) samples.

See Appendix 4 for an example calculation and Appendix 5 for typical untreated control and fortified control chromatograms

10. REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1	MEAIX018	On going	**Netzband, D.J.**, In House Laboratory Validation Of An Analytical Method For The Determination Of Residues Of AE 0317309 And Its Metabolite AE B197555 In Water Using LC/MS/MS, 2004
2	P 614 047066	MR-139/05	**Krebber, R.**, Independent Laboratory Validation Of Method AI-003-W05-01 For The Determination Of AE 0317309 And Its Metabolite AE B197555 In Water, 2005

Table 1 Analytical Method Summary Parameters

Analyte(s)	AE 0317309 and AE B197555
Extraction solvent / Technique	SPE concentration using a RP-102 cartridge
Cleanup Strategies	SPE concentration using a RP-102 cartridge
Instrument Detector Column	HPLC system: Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10A VP Controller Autosampler: Gilson 215 Liquid handler and Gilson 819 Valve Actuator Detector: Applied Biosystems API 3000 MS/MS Column: Phenomenex Prodigy 5µ C8 50 x 2.00 mm Column
Standardization Method	Multi point calibration curve (Internal standard)
Stability of Standard Solutions	Calibration standard solutions are stable for a minimum of 6 months when stored in the dark at ≤4°C
Retention times	AE 0317309 (~4 minutes) and AE B197555 (~4.6 minutes)

Appendix 1 Instrument Conditions

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 3000 LC/MS/MS System with Valco Divert Valve	
Interface:	PE Sciex Turbo Ion Spray Electrospray	
Synchronization Mode:	LC Sync	
AutoEquilibration:	Off	
Acquisition Duration:	15 min. 0 sec.	
Number of Scans:	594	
Periods in File:	2	
Acquisition Module:	Acquisition Method	
Software Version:	Analyst 1.2	
Period 1:	Period Delay:	0.00 sec.
	Scans In Period:	296
	Relative Start Time:	0.00 msec.
	Experiments in Period:	1
Period 1 Experiment 1: Duration 3.65 Minutes	Scan Type:	MRM
	Polarity:	Positive
	Scan Mode:	N/A
	Resolution Q1:	Unit
	Resolution Q3:	Low
	Intensity Threshold:	0 counts
	Smart Settling:	Off
	Settling Time:	0.0000 ms
	MR Pause:	2.0000 ms
	MCA:	No
	Step Size:	0.00 amu

LC/MS/MS Parameters (cont'd)

Acquisition Information (cont'd)

Analyte (~4.0 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0317309	363	251	500	DP	51.0	51.0
				FP	260.0	260.0
				EP	10.0	10.0
				CE	31.00	31.00
				CXP	17.00	17.00

Analyte (~4.0 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE 0317309-d_3	366	254	500	DP	51.0	51.0
				FP	260.0	260.0
				EP	10.0	10.0
				CE	30.00	30.00
				CXP	16.00	16.00

Parameter Table (Period 1 Experiment 1):	NEB:	8.0
	CUR:	10.0
	IS:	4700.0 volts
	TEM:	550° C
	CAD:	11.0

Period 2:	Period Delay:	0.00 sec.
	Scans In Period:	298
	Relative Start Time:	4.95 min.
	Experiments in Period:	1

Period 2 Experiment 1: Duration 5.0 Minutes	Scan Type:	MRM
	Polarity:	Negative
	Scan Mode:	N/A
	Resolution Q1:	Unit
	Resolution Q3:	Low
	Intensity Threshold:	0 counts
	Smart Settling:	Off
	Settling Time:	700.0000 ms
	MR Pause:	2.0000 ms
	MCA:	No
	Step Size:	0.00 amu

LC/MS/MS Parameters (cont'd)

Acquisition Information (cont'd)

Analyte (~4.6 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE B197555	267	223	500	DP	-11.0	-11.0
				FP	-50.00	-50.00
				EP	-10.00	-10.00
				CE	-12.00	-12.00
				CXP	-13.00	-13.00

Analyte (~4.6 Min.)	Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)	Parameter	Start	Stop
AE B197555 -$^{13}C_6$	273	229	500	DP	-36.00	-36.00
				FP	-140.00	-140.00
				EP	-10.00	-10.00
				CE	-16.00	-16.00
				CXP	-13.00	-13.00

Parameter Table (Period 2 Experiment 1):		
	NEB:	10.0
	CUR:	10.0
	IS:	-4100.0 volts
	TEM:	550° C
	CAD:	3.0

Auto Sampler Parameters

Autosampler Used:	Gilson 215 Autosampler
Syringe Size:	500 µL
Injection Volume:	40 µL
Pre-inject Flushes:	0
Post inject Flushes:	5
Air Cushion:	10 µL
Excess Volume:	10 µL
Sample Speed:	5.00 mL/min
Needle Level	75%
Inject Delay Time:	0.00 min
Needle Z-Direction Speed	Very Fast
Inject Time Delay	0.0 min
Loop Volume:	100 µL
Needle Flush Volume:	400 µL
Flush Speed	5.00 mL/min
Port Flush Volume	450 µL

LC/MS/MS Parameters (cont'd)

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:	5000 psi
Shutdown Time:	999.9 min.
Guard Column:	Javelin-Direct Connect Column Filter (2.1 mm i.d.)
Column Temperature:	Ambient
Column:	Manufacturer: Phenomenex
	Type: Prodigy
	Phase: C8
	Particle Size: 5 µM
	Diameter: 2.0 mm
	Length: 50 mm
Mobile Phase A:	0.1% (v/v) Acetic Acid in Water
Mobile Phase B:	850mL Acetonitrile/150mL Water + 300□L Formic Acid

Gradient Program:

Step	Time (min.)	Flow	Gradient	A(%)	B(%)	C(%)	D(%)
0	0.0	550 µL/min.	0	97.0	3.0	0.0	0.0
1	1.0	550 µL/min.	0	97.0	3.0	0.0	0.0
2	7.0	550 µL/min.	0	3.0	97.0	0.0	0.0
3	9.0	550 µL/min.	0	3.0	97.0	0.0	0.0
4	9.1	550 µL/min.	0	97.0	3.0	0.0	0.0
5	12.0	550 µL/min.	0	97.0	3.0	0.0	0.0

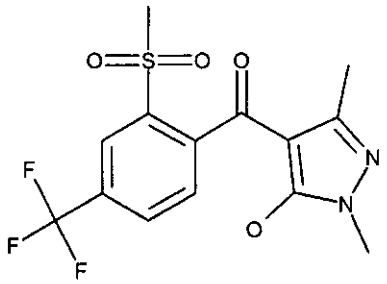
Divert Valve Program:

Step	Total Time (min.)	Divert Location
1	0.0	To Waste
2	2.0	To LC/MS
3	7.5	To Waste

Appendix 2 Structures

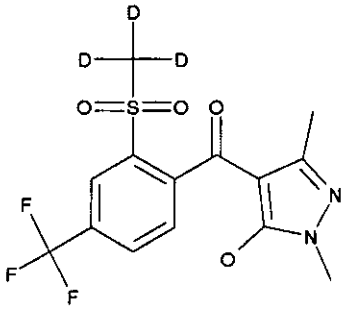
The structures for AE 0317309, its metabolite AE B197555 and the internal standards are presented below:

Code Name: AE 0317309
(Active Ingredient, Parent Molecule)



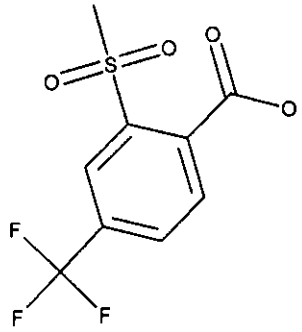
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone
CAS Number [365400-11-9]
Molecular Formula: $C_{14}H_{13}F_3N_2O_4S$
Molecular Weight: 362.33

Code Name: AE 0317309-*methylsulfonyl*-d_3
(Parent Molecule, Deuterated Internal Standard)



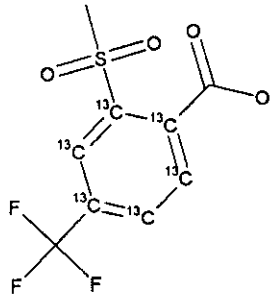
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone-methylsulfonyl-d_3
Molecular Formula: $C_{14}H_{10}D_3F_3N_2O_4S$
Molecular Weight: 365.35

Code Name: AE B197555 (or RPA2303328)
(Soil Metabolite)



CAS Name: 2-(Methylsulfonyl)-4-(trifluoromethyl)benzoic acid
CAS Number: [142994-06-7]
Molecular Formula: $C_9S0_4F_3H_7$
Molecular Weight: 268.21

Code Name: AE B197555-*phenyl* $^{13}C_6$ (or AE1345453-*phenyl* $^{13}C_6$)
(Soil Metabolite, ^{13}C Internal Standard)



CAS Name: 2-(Methylsulfonyl)-4-(trifluoromethyl)benzoic acid-phenyl $^{13}C_6$
Molecular Formula: $C_3{}^{13}C_6S0_4F_3H_7$
Molecular Weight: 274.14

Appendix 4 Example Calculation For Determination Of AE 0317309 and AE B197555 Residues

AE 0317309 and AE B197555 residues were quantified using internal standard linear regression analysis. 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 1.2), 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[1]
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

[1] As the concentration of the internal standard is the same in both the calibration standards and fortified samples, it may be omitted from these calculations.

The example shown below is for the calculation of AE 0317309 residues. AE B197555 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 AE 0317309 in the soil was calculated using the following equation,

$$\text{AE 0317309 found (ppb)} = \frac{(Y-B) \times D}{M}$$

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

Analyst software was used to calculate the amount of AE 0317309 in ppb for each sample and the percent recovery for the spiked samples.

An example calculation from the validation of AE 0317309 in tap water is described below. The complete set of data from this analytical set is summarized in a Microsoft Excel spreadsheet which is presented at the end of this section.

An example calculation for AE 0317309 from sample Tap Water LOQ 3 obtained during the validation of AE 0317309 and AE B197555 in tap water is shown below.

V_1	V_2	Native Peak Area	IS Peak Area	Y	M	B
100	5	42771.9	97689.3	0.4378	0.4951	0.0524

From the above equations:

$$\text{Dilution Factor (D)} = \frac{5}{100} = 0.05$$

$$\text{AE 0317309 found} = \frac{(0.4378-0.0524) \times 0.05}{0.4951} = 0.0389 \text{ ppb}$$

Therefore 0.0389ppb of AE 0317309 was detected in sample Tap Water LOQ 3.

The % Recovery in each sample set was calculated using the following formula:

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

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

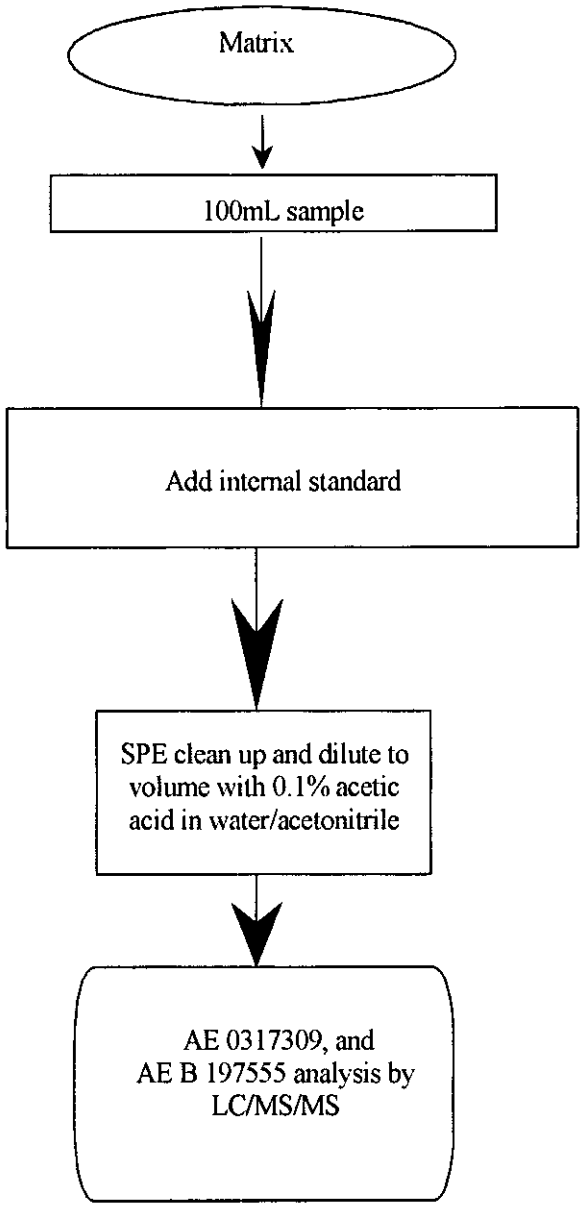
In the above example, 0.0389ppb of AE 0317309 was detected in the fortified control sample, and no apparent residue was detected in the control sample. As the sample was initially fortified with 0.05ppb AE 0317309 the % recovery was calculated using the following equation:

$$\% \text{ AE 0317309 Recovery} = \frac{(0.0389 - 0.00)}{0.05} \times 100 = 78\%$$

A Microsoft Excel spreadsheet summarizing the AE 0317309 data collected during the method validation in tap water is presented in Figure 1. The control sample, fortification sample and representative standards from this validation set are presented in Appendix 5.

Note: These example calculations were performed using rounded numbers, and therefore may be slightly different from the value shown in the raw data.

Appendix 6 Method Flow Chart



Appendix 7 Revision History

Method #	Revision	Description
AI003-W05-01	01	Method prepared on completion of validation study[1]
AI003-W05-02	02	Method updated on completion of ILV[2]