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 soil and sediment by LC/MS/MS.

The original protocol covered the analysis of AE 0317309 and AE B197555 in soil. The validation of sediment was added to the study by protocol amendment on May 23, 2005.

On completion of this study the analytical method AI-002-S05-01: "AE 0317309: Analytical Method For The Determination of AE 0317309 And Its Metabolite AE B197555 In Soil and Sediment by LC/MS/MS" was prepared.

An ILV was performed on analytical method AI-002-S05-01 (See Section 9.6), which resulted in one minor modification to the method. The modified method was assigned an analytical method number of AI-002-S05-02 and is presented in Appendix 2 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 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 January 19, 2004. The experimental phase of the study began on January 19, 2004 and concluded on June 3, 2005.

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

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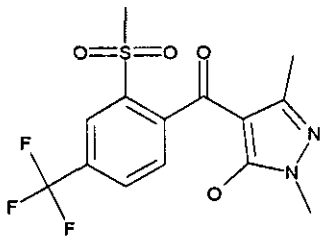
4. TEST AND REFERENCE SUBSTANCES

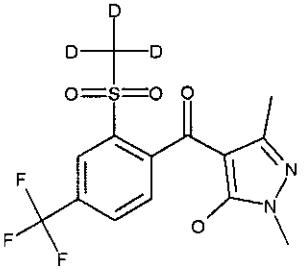
The following compounds were used as test and reference substances, and were supplied by Bayer CropScience. Neat standards were stored in a freezer at or below approximately −10°C. Standard solutions were stored in a refrigerator at approximately 4°C.

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



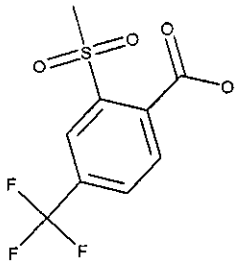
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone
CAS Number [365400-11-9]
Molecular Formula: $C_{14}H_{13}F_3N_2O_4S$
Molecular Weight: 362.32
ID No.: K-1394
Reference No.: 0324200303
Purity: 99.5%
Expiration Date: 26 October 2008
Storage Conditions: Frozen

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



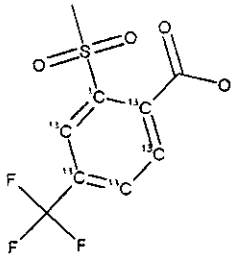
CAS Name: (5-Hydroxy-1, 3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone-methylsulfonyl-d_3
Molecular Formula: $C_{14}H_{10}D_3F_3N_2O_4S$
Molecular Weight: 365.35
ID No.: K-1261
Reference No.: 2001BRP113-37
Purity: 96.1%
Expiration Date: 11 August 2013
Storage Conditions: 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.: 1008200201
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 – SOIL AND SEDIMENT SAMPLES

The method was validated using two soil and two sediment samples. The soil samples used in this study were collected for Bayer CropScience Study Number 03BCS01: Terrestrial Field Dissipation OF AE 0317309 in Canada, 2003-2004[3].

Sample ID	Original Study	Soil type	Source Location
03BCS01-B	03BCS01	Brunisolic Gray Brown Luvisol	Ontario, Canada
03BCS01-C	03BCS01	Black Chernozem.	Manitoba, Canada

The sediment samples used in this study were collected for Bayer CropScience Study Number EBFIY003: Analysis of water and sediment samples taken from an investigation of the toxicity of fipronil to sediment dwelling organisms in the field[4].

Sample ID	Original Study	Sediment source	Source Location
LT	EBFIY003	Lake Tuscaloosa	Tuscaloosa, AL
SR-L	EBFIY003	Sandhill Research Station Lake	Columbia, SC

6. STORAGE

The untreated soil samples were stored at room temperature and the sediment was stored in a refrigerator at approximately 4°C.

7. REAGENTS AND EQUIPMENT

7.1 Reagents and General Equipment

The reagents and equipment 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 soil and sediment were determined using an Applied Bio / Sciex API-3000 LC/MS/MS system 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 Series 215 autosampler. The Applied Biosystems instrument software applications used was Analyst 1.2.

Two separate sets of LC conditions were used during the study. The soil samples were analyzed using the conditions developed during the initial development of the method. The sediment

samples, which were analyzed at a later date, were analyzed using an alternate set of conditions. The revised LC conditions resulted in both improved chromatography, and a faster run time. The same MS/MS ions were used for all analyses.

The LC conditions used for the sediment validation and MS/MS operating parameters used are outlined in Appendix I of the analytical method, which is presented in Appendix 2 of this report. The LC conditions used for the soil validation are presented after the analytical method in Appendix 2 of this report.

Chromatograms using both sets of LC conditions are presented in Appendix 1. Chromatograms 1 through 6 shows example chromatography using the initial set of LC conditions, while chromatograms 7 through 12 shows example chromatography using the modified set of LC conditions.

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 (Version 1.2) 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 techniques are described in Section 8.2 of the analytical method and an example calculation is presented in Appendix 4 of the analytical method. The analytical method is presented in Appendix 2 of this report.

Appendix 2

Analytical Method AI-002-S05-02

AE 0317309: Analytical Method for the Determination of AE 0317309 and its Metabolite AE B197555 in Soil and Sediment 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 soil and sediment, and the method validated in Bayer CropScience Study Number 04MEAIX017[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 was obtained from the method validation study.

3. PRINCIPLE

Preliminary metabolism work from metabolism studies indicated that "a marked amount" of AE 0317309 was released using an elevated temperature extraction over the more traditional approach shaking with an organic solvent mixture. Other extraction efficiency studies, while not specific to AE 0317309, also suggest that temperature is the most influential factor in improving extraction recoveries for the analytes of interest rather than solubility of the analyte in the extraction solvent. For the reasons mentioned above, the procedure selected for extracting residues of AE 0317309 and its associated metabolite AE B197555 *from soil was accelerated solvent extractor* (ASE).

The ASE accelerates traditional extraction processes by using solvents at elevated temperatures. Pressure is applied to the sample extraction cell to maintain the heated solvent in a liquid state during the extraction. After heating, the extract is flushed from the sample cell into a collection vial and is ready for analysis or additional processing.

The final quantitative detection of AE 0317309 and its metabolite AE B197555 is accomplished by LC/MS/MS. This technique will allow quantitation of all analytical targets with a high inherent specificity and without the need of derivitization for the more polar analytes.

AE 0317309 and its associated metabolite AE B197555 are extracted from soils using an Accelerated Solvent Extractor (ASE) with 65:35 acetonitrile/water at 100°C / 1500 psi pressure. Following extraction, the extracts are fortified with an isotopic internal standard containing AE 0317309-d_3 and AE B197555-$^{13}C_6$, an aliquot is evaporated to reduced volume on a Zymark Turbovap, 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.

- Solo Cone Water Cups, 4 oz. (No.: 4R)
- VWR SuperClear Centrifuge Tubes, Polypropylene (Cat. No.: 21008-169)
- Dionex 33 mL Extraction Cell, Assembled (Part No.: 0487673)
- Dionex Cellulose Filters for Extraction Cell Caps (Part No.: 049458)
- Dionex Cellulose Filter Insertion Tool (Part No.: 049495)
- VWR Pyrex® Brand volumetric pipets, glass class A (Assorted Volumes)
- Rainin Microman® Classic positive-displacement pipettes (Cat. No.: M-50, and M-250)
- Dionex 60 mL Clear Collection Vials (Part No.: 048784)
- Dionex Accelerated Solvent Extractor (Model ASE 200)
- VWR Pyrex® Brand graduated cylinder (Cat. No.: 24760-100)
- VWR Pyrex® Brand volumetric flasks, glass class A (Assorted Volumes)
- VWR Pyrex® Brand glass funnels
- 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)
- 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 and/or Heated Nebulizer Interface.
- Shimadzu LC-10AD VP HPLC pumps (two), Shimadzu SCL-10A VP Controller with a Gilson 215 Liquid handler and Gilson 819 Valve Actuator.
- VICI Cheminert Valve and 2 position actuator Controller.
- Zymark TurboVap II
- Applied Separations 200mg/3mL RP-102 Resin Spe-ed SPE Cartridges (Catalog # 4208)
- 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.

- Varian Hydromatrix bulk material (Part. No.: 0019-8003)
- Sand, Standard Ottawa, EM Science (VWR Cat. No.: EM-SX0070-1)
- 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)
- Certified analytical reference standards of AE 0317309 and AE B197555
- Certified internal standards of AE 0317309-methylsulfonyl-d_3 and AE B197555-phenyl $^{13}C_6$

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 5°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 6 months when stored in the dark at ≤-18°C.

6.2 Fortification Standard Solutions

Prepare a 10 µg/mL solution containing a mixture of AE 0317309 and 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 standard 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 standard 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 6 months when stored in the dark at ≤5°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 and 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: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.

The calibration standard solutions are stable for a minimum of 6 months when stored in the dark at ≤5°C.

7. ANALYTICAL PROCEDURE FOR ANALYSIS OF SOIL AND SEDIMENT

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 Sample Preparation

Samples of soil and sediment should be thoroughly homogenized and stored frozen until sampled for extraction.

7.2 Extraction

NOTE: *This method uses internal standards to determine the concentrations of AE 317309 and AE B197555 present in soil. If the concentration of these components are outside the range of the appropriate calibration curve the analyses will have to be repeated using either a reduced sample weight or by diluting the ASE extract prior to the addition of the internal standard. If a further dilution is made to the ASE extract adjust the concentration of internal standard*

added in step 7.2.9 so that the final concentration of internal standard present in the final sample is 2ppb.

7.2.1 Weigh an aliquot of soil or sediment (maximum of 25± 0.1 g) into a disposable centrifuge tube (50 mL). ♦

7.2.2 Add 3.0 ± 0.1 grams of hydromatrix, cap the tube and manually shake the sample to thoroughly mix the contents. If the sample contains significant quantities of water (such as sediment samples) an additional 3 ± 0.1 grams of hydromatrix may be added.

7.2.3 Assemble two clean caps for a 33 mL Dionex extraction cell using a stainless steel frit for the first and a cellulose filter for the second. Attach the first cap to the extraction cell.

NOTE: *A disposable funnel may be made by cutting the bottom out of a Solo cone water cup with a pair of scissors.*

7.2.4 Transfer the soil (or sediment) / hydromatrix mixture from step 7.2.2 into the extraction cell using a disposable paper funnel. Gently tap the extraction cell on the lab bench to compact the sample.

NOTE: *The samples should be fortified using a class A glass volumetric pipette or an accurately calibrated variable volume micropipettor.*

7.2.5 *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). The samples should be fortified at the top of the soil column contained in the extraction cell.

7.2.6 Top off the extraction cell with sand to fill the void volume. The extraction cell should be completely filled. Screw the second assembled cap onto the top of the extraction cell. Do not allow sand to get into the screw threads. Hand tighten both cell caps snuggly.

7.2.7 Load the extraction cells onto the ASE system along with appropriate number of 60 mL collection vials.

7.2.8 Program the ASE extraction unit with the following parameters and extract the samples:

ASE 200 Parameters	Setting
Preheat (min.):	0 minutes
Heat (min.):	5 minutes
Static time (min.):	15 minutes
Flush Volume (% Vol.):	80%
Purge Time (sec.):	150 sec.
Cycles (#):	2
Pressure (psi):	1500 psi
Temperature (°C):	100° C
Extraction Solvent (%A):	65%: Acetonitrile
Extraction Solvent (%B):	35%: Deionized water

NOTE: *The sample extracts may be allowed to stand in the collection vials overnight prior to concentration and SPE clean up. For convenience the analyst may perform the concentration and SPE clean up the next working day.* ♦

7.2.9 Add by pipet 250µL of the 0.1µg/mL internal standard solution prepared in 10% acetonitrile/90% 0.1% acetic acid in deionized water. (see Section 6.3 Internal Standard Solutions) to the contents of each ASE sample collection vial (volume of sample extract ~50mL).

7.2.10 Transfer a ~20mL aliquot from the ASE sample collection vial to a 200 mL Zymark evaporation flask.

7.2.11 Evaporate to ~5mL using a Turbovap® II evaporator set to a temperature of 50°C.

7.2.12 Add 60µL of formic acid to the contents of the Zymark evaporation flask and place in an ultrasonic bath for 2 – 3 minutes.

7.3 SPE Clean Up

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

7.3.2 Transfer the contents of the Zymark evaporation flask to the cartridge by pipet (~1 drop/2 sec).

7.3.3 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.

7.3.4 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.

7.3.5 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.

7.3.6 Add ~1.0 mL of 50:50 acetonitrile/methanol to the cartridge. Apply positive pressure and push the solvent onto the cartridge. Take precautions to insure that no eluent is lost. *Positive pressure can be applied via a hand held nitrogen line.* Vent the pressure and allow the cartridge to soak for 1-2 minutes. Reapply pressure and elute all solvent (~1drop/second) into a 10mL test tube with a screw top lid.

7.3.7 Add ~4mL 0.1% (v/v) acetic acid: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. ♦

8. ANALYSIS

8.1 Sample Analysis

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

Inject a 30 µl aliquot of each test sample (or fortified sample matrix) from step 7.3.7 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 LC/MS/MS Standard Calibration and Residue Calculations

Standardize the LC/MS/MS response under the conditions outlined in Appendix 1 by injecting a 30 µL aliquot of each LC/MS/MS calibration solution interspersed with samples.

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
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 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,

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

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume}(V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

Where: W = Sample weight
V_1 = 50 mL
V_2 = 20 mL
V_3 = 5 mL

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 is 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.*

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

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

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

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

Note: See Appendix 5 for typical untreated control and fortified control chromatograms

10. REFERENCES

No.	Doc. No.	Report No.	**Author(s).** Title. Year.
1	04MEAIX017	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 Soil Using LC/MS/MS
2	03BCS01	MEAIM004	**Belyk, M.**, Terrestrial Field Dissipation Of AE 0317309 in Canada, 2003 - 2004
3	P 611050012	MR-112/05	**Brumhard, B.**, Independent Laboratory Validation Of Method AI-002-S05-01 For The Determination Of AE 0317309 And Its Metabolite AE B197555 In Soil and Sediment by LC/MS/MS, 2005

Table 1 Analytical Method Summary Parameters

Summary Parameters for the Analytical Method Used for the Quantitation of AE 0317309 and AE B197555 Residues in Soil. (TABLE B.1.1.)	
Method ID	AI-002-S05-02
Analyte(s)	AE 0317309 and AE B197555
Extraction solvent / Technique	Dionex 200 Accelerated Solvent Extraction (ASE) using an extraction solvent of 65% acetonitrile/35% water. Two extraction cycles were performed at 100°C/1500psi
Cleanup Strategies	SPE Clean-Up 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 (~3.4 minutes) and AE B197555 (~3.8 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

Appendix I (continued)

Acquisition Information (cont'd)

Analyte (~3.4 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 (~3.4 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

Appendix I (continued)

Acquisition Information (cont'd)

Analyte (~3.8 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 (~3.8 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:	30 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

Appendix I (continued)

Acquisition Information (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: C_8 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 + 300µL 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	4.0	550 µL/min.	0	7.0	93.0	0.0	0.0
3	13.0	550 µL/min.	0	7.0	93.0	0.0	0.0
4	13.0	550 µL/min.	0	97.0	3.0	0.0	0.0
5	15.0	550 µL/min.	0	97.0	3.0	0.0	0.0

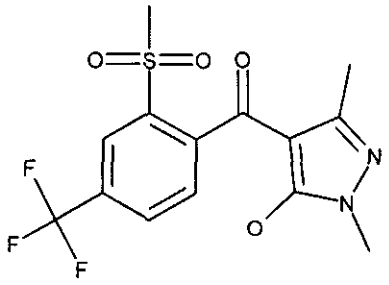
Divert Valve Program:

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

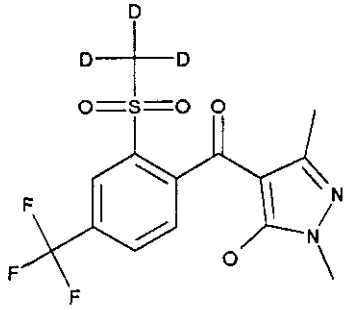
The structures for AE 0317309 and its metabolite AE B197555 are presented below:

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



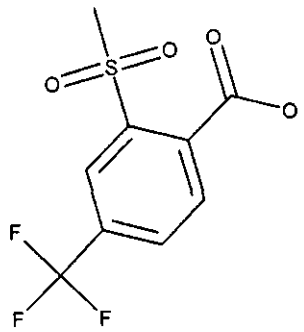
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone
CAS Number [365400-11-9]
Molecular Formula: $C_{14}H_{13}F_3N_2O_4S$
Molecular Weight: 362.33

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



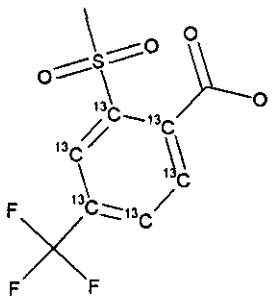
CAS Name: (5-Hydroxy-1,3-dimethyl-1H-pyrazol-4-yl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methanone-methylsulfonyl-d_3
Molecular Formula: $C_{14}H_{10}D_3F_3N_2O_4S$
Molecular Weight: 365.35

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

An example calculation for AE 0317309 from sample 05AI-SLLOQ-006, which was analyzed during the method validation study, is shown below. This sample was fortified with 0.5ppb AE 0317309 and AE B197555. The chromatogram used in this example is presented in Appendix 5 (Chromatogram 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 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,

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

$$\text{Where Dilution Factor (D)} = \frac{\text{Initial volume}(V_1)}{\text{Initial sample wt. (W)}} \times \frac{\text{Final dilution volume } (V_3)}{\text{Aliquot taken } (V_2)}$$

W	V_1	V_2	V_3	Native Peak Area	IS Peak Area	Y	M	B
20g	50mL	20mL	5mL	5900.5	17591.4	0.3354	0.5684	0.0100

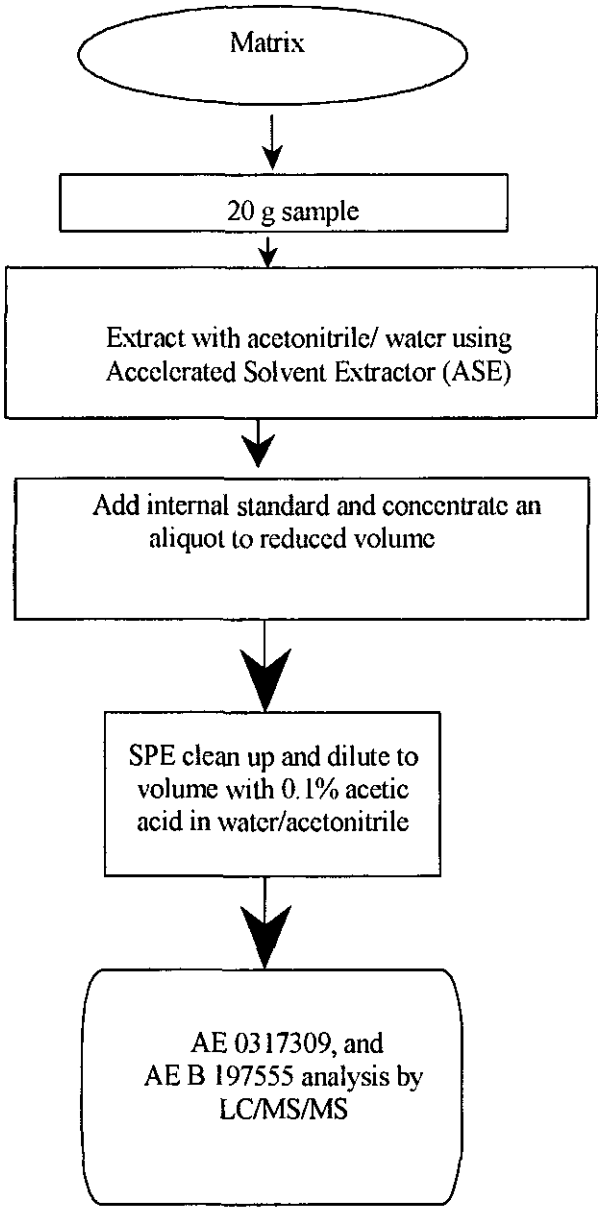
From the above equations:

$$\text{Dilution Factor (D)} = \frac{50}{20} \times \frac{5}{20} = 0.625$$

$$\text{AE 0317309 found} = \frac{(0.3354-0.0100) \times 0.625}{0.5684} = 0.3578 \text{ ppb}$$

Therefore sample 05AI-SLLOQ-006 contains 0.3578ppb AE 0317309

Appendix 6 Method Flow Chart



Appendix 7 Revision History

Method #	Revision	Description
AI002-S05-01	01	Method prepared on completion of validation study[1]
AI002-S05-02	02	Method prepared on completion of ILV

Alternate LC conditions used in soil analysis

The LC conditions used for the soil validation are presented below. Using these conditions the approximate retention times of AE 0317309 and AE B197555 are 8.2 and 10.6 minutes respectively.

Pump Used:	Two Shimadzu LC-10AVP (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:	Waters
	Type:	SymmetryShield
	Phase:	RP8
	Particle Size:	5 µM
	Diameter:	3.0 mm
	Length:	150 mm
	Pore Size:	100 Å
	Mobile Phase A:	Acetonitrile
	Mobile Phase B:	0.1% (v/v) Acetic Acid

Gradient Program:

Step	Total Time (min.)	Flow	Gradient	A(%)	B(%)	C(%)	D(%)	TE#1	TE#2
0	0.00	500 µL/min.	0	20.0	80.0	0.0	0.0	open	open
1	1.00	500 µL/min.	0	20.0	80.0	0.0	0.0	open	open
2	8.00	500 µL/min.	0	90.0	10.0	0.0	0.0	open	open
3	12.50	500 µL/min.	0	90.0	10.0	0.0	0.0	open	open
4	12.60	500 µL/min.	0	20.0	80.0	0.0	0.0	open	open
5	15.00	500 µL/min.	0	20.0	80.0	0.0	0.0	open	open

Divert Valve Program:

Step	Total Time (min.)	Divert Location
1	0.0	To Waste
2	6	To LC/MS
3	15.0	To Waste