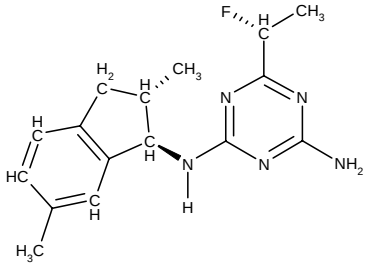
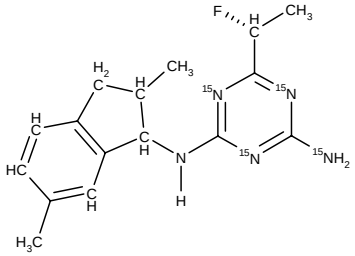
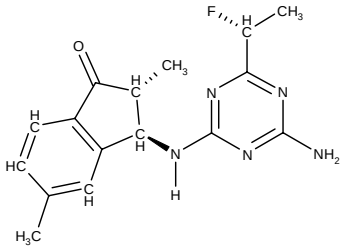
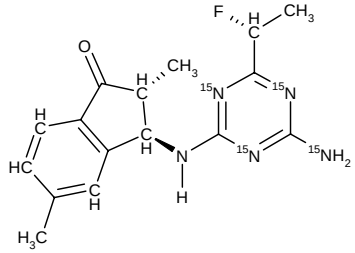
3. PRINCIPLE

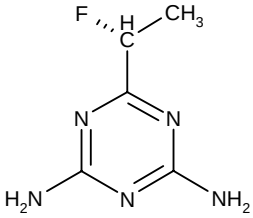
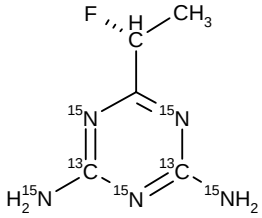
AE1170437 and its associated metabolites are extracted from soil and sediment by adding 80:20 (*v/v*) acetonitrile: water and using microwave extraction. After extraction, the mixture is fortified with isotopically labeled internal standards of AE1170437 and its metabolites and centrifuged. The sample is diluted prior to analysis with HPLC grade water. Detection is achieved by tandem mass spectrometry (LC/MS/MS). Quantification is based on the use of internal standards and comparison of peak areas with those of known standards. A method flow-chart is presented in Appendix 2.

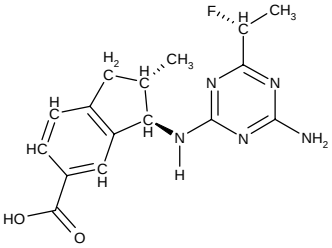
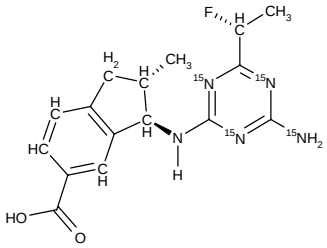
4. COMPOUNDS

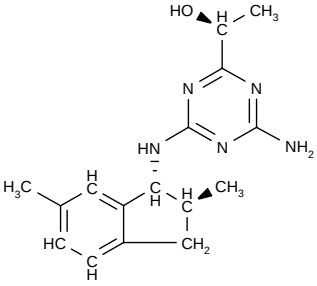
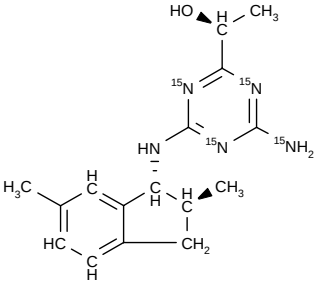
The structures for AE1170437, its metabolites and the associated internal standards are presented below:

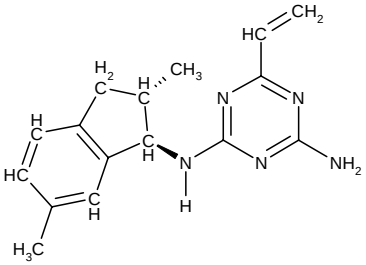
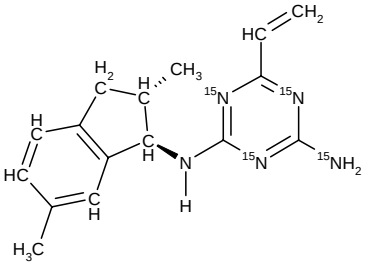
Code Name:	AE1170437 (Parent)	AE1170437-isomer mix-triazine-$^{15}N_4$ (Internal standard for AE1170437)
Structure		
Chemical Name:	N-[(1R,2S)-2,3-Dihydro-2,6-dimethyl-1H-inden-1-yl]-6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine	
Molecular Weight:	301.4	305.3
Molecular Formula:	$C_{16}H_{20}FN_5$	$C_{16}H_{20}F^{15}N_4N$

Code Name:	AE2158968 (AE1170437 Triazine-indanone, metabolite)	AE2158968-triazine-$^{15}N_4$ (Internal standard for AE1170437 Triazine-indanone)
Structure		
Chemical Name:	*N*-[(1*R*,2*S*)-2,3-Dihydro-2,6-dimethyl-3-oxo-1*H*-inden-1-yl]-6-[(1*R*)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine	
Molecular Weight:	315.3	319.3
Molecular Formula:	$C_{16}H_{18}FN_5O$	$C_{16}H_{18}F^{15}N_4NO$

Code Name:	AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine), metabolite	1-Fluoroethyl triazinediamine-$^{15}N_5$,$^{13}C_2$ (Internal standard for AE1170437 Diaminotriazine)
Structure		
Chemical Name:	6-[(1*R*)-1-Fluoroethyl]-1,3,5-triazine-2,4-diamine	
Molecular Weight:	157.1	164.1
Molecular Formula:	$C_5H_8FN_5$	$C_3H_8F^{15}N_5{}^{13}C_2$

Code Name:	AE2158969 (AE1170437 Carboxylic Acid, metabolite)	AE2158969-triazine-$^{15}N_4$ (Internal standard for AE1170437 Carboxylic Acid)
Structure		
Chemical Name:	(2*S*,3*R*)-3-[[4-Amino-6-[(1*R*)-1-fluoroethyl]-1,3,5-triazin-2-yl]amino]-2,3-dihydro-2-methyl-1*H*-indene-5-carboxylic acid	
Molecular Weight:	331.3	335.3
Molecular Formula:	$C_{16}H_{18}FN_5O_2$	$C_{16}H_{18}F^{15}N_4N\ O_2$

Code Name:	AE2300077 (AE1170437 Hydroxyethyl, metabolite)	AE2300077-triazine-$^{15}N_4$ (Internal standard for AE1170437 Hydroxyethyl)
Structure		
Chemical Name:	(1S)-1-(4-amino-6-{[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]amino}-1,3,5-triazin-2-yl)ethanol	
Molecular Weight:	299.4	303.3
Molecular Formula:	$C_{16}H_{21}N_5O$	$C_{16}H_{21}{}^{15}N_4N\ O$

Code Name:	BCS-AA10201 (AE1170437 Olefin), metabolite	BCS-AA10201-triazine-$^{15}N_4$ (Internal standard for AE1170437 Olefin)
Structure		
Chemical Name:	N-[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]-6-vinyl-1,3,5-triazine-2,4-diamine	
Molecular Weight:	281.4	285.3
Molecular Formula:	$C_{16}H_{19}N_5$	$C_{16}H_{19}{}^{15}N_4N$

5. INSTRUMENTS, EQUIPMENT, AND SUPPLIES

Use as a guide; equivalent or better apparatus may be substituted.

- Sciex API 4000 LC/MS/MS System (Applied Biosystems) With Analyst 1.4.1 or higher software
- Sciex TurboIonSpray Electrospray interface
- Two Shimadzu HPLC Pumps, LC-10ADvp (with low volume high pressure mixing)
- Shimadzu SCL-10AVP pump controller
- PerkinElmer Series 200 autosampler
- Balance for weighing analytical standards: Accuracy ± 0.1 mg, Mettler AT 201
- Balance for weighing samples: Accuracy ± 0.1 g, Precisa 1000C - 3000D
- Weighing spatulas
- Centrifuge, Beckman Allegra 6 or equivalent
- Ethos E Microwave or equivalent
- Disposable pipettes
- Glass “Class A” graduated cylinders, pipettes, Gastight® micro-syringe, and volumetric flasks
- Micropipette, Eppendorf with disposable tips or equivalent
- Nalgene® Polypropylene Wide-Mouth Bottles 4-oz, (Fisher Scientific, Cat. No. 02-893A)
- Disposable, 1"-long, 5/16"-diameter magnetic stir bars (Fisher Scientific, Cat. No. 1451394)
- HPLC vials and caps
- Synergy 4µ Fusion-RP HPLC column, (250 x 2.0 mm, 4 µm, 80A pore size) Phenomenex, Cat. No. 00G-4424-B0
- Upchurch, ultra-low volume, inline pre-column filter, catalog # A 318, with A 102x0.5 µm frits

6. REAGENTS

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

- Methanol (OPTIMA) (Fisher Scientific Cat. No. A454-4)
- Deionized Water filtered through a Milli-Q water system or Water (OPTIMA) (Fisher Scientific, Cat. No. W7-4)
- Formic acid (98%, for mass spectroscopy, Fluka, Cat. No. 94318)
- Acetonitrile (OPTIMA) (Fisher Scientific, Cat. No. A996-4)
- Certified analytical reference standards of AE1170437 and its metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201) and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine)
- Certified internal standards of AE1170437-isomer mix-triazine-$^{15}N_4$, AE2158968-triazine-$^{15}N_4$, 1-Fluoroethyl triazinediamine-$^{15}N_5$,$^{13}C_2$, AE2158969-triazine-$^{15}N_4$, AE2300077-triazine-$^{15}N_4$, BCS-AA10201-triazine-$^{15}N_4$

7. PREPARATION OF ANALYTICAL STANDARDS

Use class "A" volumetric pipettes or Teflon-seal plunger, micro-syringes to make standards. The following is an example of a procedure to follow in preparing standard solutions. Alternate or additional standards of appropriate concentration and volume may be prepared as needed. The “~” symbol indicates approximately.

All the standard solutions must be stored in amber glass bottles. Stock solutions will be stored in a freezer at < -10 °C when not in use. Standard solutions will be stored in a refrigerator at ~ 4 °C when not in use. Solutions should be allowed to warm to room temperature prior to use.

7.1 Primary Native and Isotopically Labeled Stock Standard Solutions

Transfer ~0.0100 (or 0.0050) g (corrected for purity) each of native and isotopically labeled AE1170437 and its metabolites into separate 100 (or 50) mL volumetric flasks and dilute to volume with methanol. Cap and mix by inversion. The concentration of these stock standards is ~100,000 ng/mL.

Note: Ensure complete dissolution of neat standards while preparing stock solutions since solubility varies markedly among the analytes.

7.2 Fortification Standard Solutions

1. LOQ Spike solution: Transfer a 500 µL aliquot of each of the 100,000 ng/mL native AE1170437 and its metabolites stock solutions (Section 7.1) into a 100 mL volumetric flask. Dilute to 100 mL with acetonitrile. The concentration of this solution is 500 ng/mL. 15 g soil or sediment sample is fortified to 1.5 ng/g (LOQ) by the addition of 45 µL of this 500 ng/mL solution.

2. 10XLOQ Spike solution: Transfer a 5000 µL aliquot of each of the 100,000 ng/mL native AE1170437 and its metabolites stock solutions (Section 7.1) into a 100 mL volumetric flask. Dilute to 100 mL with acetonitrile. The concentration of this solution is 5000 ng/mL. 15 g sample is fortified to 15 ng/g (10XLOQ) by the addition of 45 µL of this 5000 ng/mL solution.

3. Internal Standard (IS) spike solution: Transfer a 2500 µL aliquot of each of the 100,000 ng/mL isotopically labeled AE1170437 and its metabolites stock solutions (Section 7.1)

into a 100 mL volumetric flask. Dilute to 100 mL with acetonitrile. The concentration of this solution is 2500 ng/mL. The 30 mL extract solvent is fortified to 5 ng/mL by the addition of 60 µL of this 2500 ng/mL solution.

Name of Fortification Standard	Concentration of Stock Solution Used for Dilution (ng/mL)	Aliquot Taken (µL)	Dilution Volume (mL)	Concentration of New Solution (ng/mL)
LOQ Spike Solution	100,000 (individual stock solution)	500	100	500 (native mixed)
10X LOQ Spike Solution		5000		5,000 (native mixed)
IS Spike Solution		2500		2,500 (IS mixed)

7.3 Calibration Standard Solutions

Prepare the calibration standards that contain both native and isotopically labeled internal standards by following the dilution scheme provided in the table below (Other concentrations may be prepared as needed). For example, to prepare a mixed calibration standard containing 5.0 ng/mL of native analytes and 0.5 ng/mL of isotopically labeled analytes (last solution in the table below), take 100 µL of 5,000 ng/mL mixed native standard solution (Section 7.2.2) and place it in a 100-mL volumetric flask. Then, take 20 µL of 2,500 ng/mL mixed isotopically labeled internal standard solution (Section 7.2.3) and add to the same volumetric flask. Bring volume to the mark with 8% acetonitrile in water. Cap volumetric flask and mix by inversion.

Type of Standard	Concentration of Standard Solution Used for Dilution (ng/mL)	Aliquot Taken (µL)	Dilution Volume (mL)	Concentration of New Mixed Standard (ng/mL)
Mixed Native Mixed IS	0.0 2,500	0 20	100	0.0 0.5
Mixed Native Mixed IS	500 2,500	10 20	100	0.05 0.5
Mixed Native Mixed IS	500 2,500	20 20	100	0.1 0.5
Mixed Native Mixed IS	5,000 2,500	10 20	100	0.5 0.5
Mixed Native Mixed IS	5,000 2,500	20 20	100	1.0 0.5
Mixed Native Mixed IS	5,000 2,500	100 20	100	5.0 0.5

The standard solutions are stable for a minimum of one month when stored at ~ 4 °C in the dark.

Representative calibration curves for each of the analytes are presented in Appendix 3.

8. ANALYTICAL PROCEDURE FOR ANALYSIS OF SOIL AND SEDIMENT

A method flow chart is presented in Appendix 2, and a summary of the analytical method parameters is presented in Table 1.

8.1 Sample Preparation

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

8.2 Laboratory Fortified Sample Preparation

Sample fortification is performed by adding a certain amount of a fortification solution to 15 g of soil/sediment. For example, fortification at the LOQ of 1.5 ng/g would involve addition of 45 µL of 500 ng/mL mixed native fortification solution (Section 7.2.1) to 15 g of soil/sediment. Allow at least 15 minutes for the standard to soak into the soil/sediment and for solvent to evaporate before the samples are extracted. Procedural recovery samples should also be fortified with isotopically labeled internal standards after the microwave extraction step (see procedure in Section 8.3).

8.3 Extraction

NOTE: This method uses internal standards to determine the concentrations of AE1170437 and its metabolites present in soil and sediment. If the concentrations 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 the sample extract volume may be increased to dilute the sample and a corresponding increase in internal standard is added to the diluted extract in step 10 below.

Alternatively, a good estimate is obtained just by diluting the final extract without addition of more IS solution, provided the IS area is not reduced so much as to be unreliable, or not less than the native area corresponding to the LOQ. The required dilution before addition of IS solution may then be determined based on that estimate to provide a final result.

1. Weigh 15 ± 0.1 grams of soil or sediment into a 125-mL glass jar with a screw cap lid.

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

3. Add 30 mL of 80:20 (*v/v*) acetonitrile: water to each jar, cap and shake vigorously to break up any large soil aggregates.

4. Remove the cap and place a disposable magnetic stir bar into the glass jar. Loosely attach the lid to the glass jar. **NOTE: Over tightening the lids may cause a pressure build up inside the jar, resulting in a potential explosion hazard.**

5. Place the jars in the microwave, evenly spaced around the center of the carousel.

6. Insert the thermo-well into the untreated control sample and insert the fiber optic temperature probe into the thermo-well.

7. With the microwave door open, turn on the manual magnetic stirrer control and check to see that the stir bars are turning but without splashing the samples. The manual stirrer will override any program stirring rates.

8. Close the microwave door, and program the microwave with the following method:

Ethos E Microwave Program Parameters

Nr Step Number	t Time Duration	T1* Temperature set point at end of step	E Power Limit (to maintain/ control temperature)	Comments
1	10 min.	60 °C	≤350 W	Ramp from ambient to 60 °C
2	10 min.	60 °C	≤350 W	Maintain 60 °C

* Parameter T1 refers to the fiber optic probe temperature control parameter.

Ventilation time:	1 minute
QP limit:	60-80% (Shut off limit in case of vapors in oven becoming too concentrated)
Stirrer (speed setting):	Value not used. Manual control overrides program setting.
Rotor control:	On (Rotor rotation is on)
Twist control:	On (Rotor rotates clockwise and then counterclockwise to keep probe cable from twisting)

9. Extract the samples using the above method. On completion of the extraction cycle, allow the glass jars to cool.

10. Remove the jars from the microwave oven and fortify each sample with isotopically labeled internal standards (IS). Cap the bottles and shake vigorously for about 10 seconds to mix IS solution with the extract. The concentration of IS in the final extract for analysis should be approximately equal to the concentration of IS in the calibration standards. For example, fortification of 60 µL of 2500 ng/mL mixed IS solution (Section 7.2.3) to a 30 mL sample would give 5 ng/mL concentration in the extract (note that the extraction volume is 30 mL and there will be a 10X dilution in following steps, so the IS concentration will be 0.5 ng/mL in the final sample). The calibration standards contain 0.5 ng/mL IS.

11. Pipette 1.5 mL of the supernatant from the sample jar to an HPLC vial after the sample has settled for several minutes. Centrifuge the HPLC vial for 10 minutes at ~3500 rpm.

12. Take 100 µL of supernatant directly from the sample vial right after the centrifugation and place in another HPLC vial containing 900 µL of water. Cap the second HPLC vial and mix contents thoroughly using a vortex. The extract is ready for LC/MS/MS analysis. Store the extract in a refrigerator set at ~ 4 °C if analysis is not performed on the same day of extraction.

13. After removing 100 µL of supernatant from the centrifuged HPLC sample vial in step 12, store this HPLC vial in a refrigerator set at ~ 4 °C for reanalysis if needed. If reanalysis is needed, perform the procedure given in step 12.

9. LC/MC/MC ANALYSIS

9.1 Liquid Chromatographic Conditions

Column:	Synergy Fusion-RP, 250 x 2.0 mm, Particle size 4 µm, pore size 80Å
Column temperature:	Ambient
In-line filter:	Upchurch, ultra-low volume, inline pre-column filter, with A102x0.5 µm frits.
Mobile phase:	A: 0.05% formic acid in HPLC grade water B: 0.05% formic acid in methanol
Flow rate:	0.20 mL/min
Injection volume:	40 µL (adjust volume as needed for acceptable sensitivity)

Gradient program:

Gradient Table with percentages and flow rates listed as they are at the start of each step:

Time (min)	Flow (mL/min)	%A	%B	Step Description
0.01	0.20	60	40	(initial condition)
0.20	0.20	60	40	(start linear ramps)
1.00	0.20	35	65	
4.00	0.20	30	70	
6.00	0.20	20	80	
6.50	0.20	5	95	(end linear ramps)
9.90	0.20	5	95	(end plateau)
10.00	0.20	60	40	(start equilibration)
14.00	Stop			(end run)

9.2 Compound Identification

The analytes are identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under same conditions. Some compound analysis and identification parameters are listed as follows:

Compound	*Approx.* RT, min	Q1, *amu*	Q3, *amu*	DP, V	CE, V	CXP, V
AE1170437	10.5	302	158	61	17	15
AE1170437-$^{15}N_4$	10.5	306	162	61	17	15
AE1170437 Diaminotriazine	4.9	158	138	56	21	10
AE1170437 Diaminotriazine-$^{15}N_5$,$^{13}C_2$	4.9	165	145	56	21	10
AE1170437 Hydroxyethyl	6.2	300	156	86	25	12
AE1170437 Hydroxyethyl-$^{15}N_4$	6.2	304	160	86	25	12
AE1170437 Carboxylic Acid	8.2	332	158	92	29	16
AE1170437 Carboxylic Acid-$^{15}N_4$	8.2	336	162	92	29	16
AE1170437 Triazine-indanone	8.4	316	158	80	30	11
AE1170437 Triazine-indanone-$^{15}N_4$	8.4	320	162	80	30	11
AE1170437 Olefin	9.0	282	138	93	23	14
AE1170437 Olefin-$^{15}N_4$	9.0	286	142	93	23	14

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensities.
All the above compounds are detected in positive polarity mode.
DP: Declustering Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential

9.3 Sample Analysis

AE1170437 and its metabolites were analyzed by LC/MS/MS using isotopic internal standards.

Inject a 40 µL aliquot of each test sample (or fortified sample matrix) in Section 8.3 onto the LC/MS/MS under the conditions presented in Appendix 4. 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 2 to 3 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.

Example chromatograms are shown in Appendix 5.

9.4 LC/MS/MS Standard Calibration and Residue Calculations

The example calculation displayed below is from the method validation. Alternate calculation procedures appropriate to the reporting requirements may be substituted.

Standardize the LC/MS/MS response under the conditions outlined in Appendix 4 by injecting an aliquot of each LC/MS/MS calibration solution. Standards should be interspersed with samples or bracket sample runs to compensate for any minor change in instrument response.

To generate calibration curves for AE1170437 and its metabolites, a minimum of five standards over a range of concentration levels should be included with a set of samples. To bracket samples with residues near the LOQ, the lowest standard run will be between the limit of quantitation (LOQ) and limit of detection (LOD).

Linear regression coefficients should be calculated for the ratio of analyte to its corresponding internal standard area or height plotted versus the ratio of analyte to its corresponding internal standard concentration in the calibration standards. The data from the analytical standards should then be fit to the linear model,

$$y = Ax + B$$

x = Concentration ratio of the reference standard in ng/mL to the internal standard concentration. (As the reference standards and samples contain the same internal standard concentrations it may be omitted from the calculation by substituting a value of one in both standards and samples)
y = Response ratio

The equation to be used to estimate the residues in the samples is:

$$E = \frac{(y - B)}{A} \times D$$

Where: E = concentration of analyte in sample in parts per billion (ppb or ng/g)
y = ratio of analyte response (area or height) to internal standard response (area or height)
B = intercept from linear regression analysis
A = slope from linear regression analysis (area ratio per conc. ratio)
D = dilution factor (e.g. 20 mL/g in this method)

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

Where: W = 15 g
V1 = 30 mL
V2 = 0.1 mL
V3 = 1.0 mL

For a better estimation of any residues between the lowest standard and the LOD, the linear regression may be forced through zero.

An example of calculation is shown in Appendix 6.

9.5 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 and 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 1.5 ppb 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.

10.3 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 in positive polarity. The following alternate ions are suggested, and the spectra for each of the analytes are presented in Appendix 5.

Compound	Q1,*amu*	Q3,*amu*	DP, V	CE, V	CXP, V
AE1170437	302	138	61	37	14
AE1170437 Diaminotriazine	158	85	56	27	10
AE1170437 Hydroxyethyl	300	138	86	35	12
AE1170437 Carboxylic Acid	332	138	92	43	14
AE1170437 Triazine-indanone	316	138	80	37	11
AE1170437 Olefin	282	145	93	33	14

Note: All the above compounds are detected in positive polarity mode.
DP: Declustering Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential

10.4 Time Considerations

A set of fourteen samples can be weighed and prepared for analysis within 4 hours. The samples are analyzed overnight and the data are processed the following day.

11. SAFETY

All available appropriate Material Safety Data Sheets (MSDS) should be available to the study personnel during the conduct of the method. General laboratory safety precautions should be taken.

12. REFERENCES

1. Xu, T., R.J. Seymour, In House Laboratory Validation of an Analytical Method for the Determination of Residues of AE1170437 and its metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201), and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine) in Soil and Sediment Using LC/MS/MS, Bayer Study Number: RADHP046.

2. Schmeer, K., K.-H. Löhrwald, Independent Laboratory Validation of Method DH-002-S06-01 for the Determination of AE1170437 and its Metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201) and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine) in Soil and Sediment Using LC-MS/MS, bayer Study Number: MR-07/253

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

Summary Parameters for the Analytical Method Used for the Quantitation of AE1170437 and its metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201), and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine) in Soil and Sediment		
Method ID	DH-002-S06-01	
Analytes	AE1170437 and its metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201), and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine)	
Extraction solvent / Technique	Microwave extraction using 80% acetonitrile/20% water	
Cleanup Strategies	Dilution	
Instrument Detector Column	- Applied Biosystems API 4000 MS/MS with Sciex TurboIonSpray Electrospray interface - Shimadzu LC-10AD VP HPLC pump with Shimadzu SCL-10AVP pump controller - PerkinElmer Series 200 autosampler - Synergy 4µ Fusion-RP HPLC column, (250 x 2.0 mm, 4 µm, 80Å pore size) with - Upchurch, ultra-low volume, inline pre-column filter with A 102x0.5 µm frits	
Standardization Method	Multi point calibration curve (Isotopically labelled Internal standard)	
Stability of Standard Solutions	Stock standard solutions are stable for a minimum of 3 months when stored in the dark at ≤ -10 ºC Fortification and calibration standard solutions are stable for a minimum of 1 month when stored in the dark at ~4 °C	
Approximate Retention times	AE1170437 AE1170437 Carboxylic Acid AE1170437 Triazine-indanone AE1170437 Hydroxyethyl AE1170437 Olefin AE1170437 Diaminotriazine	10.5 min. 8.2 min. 8.4 min. 6.2 min. 9.0 min. 4.9 min.

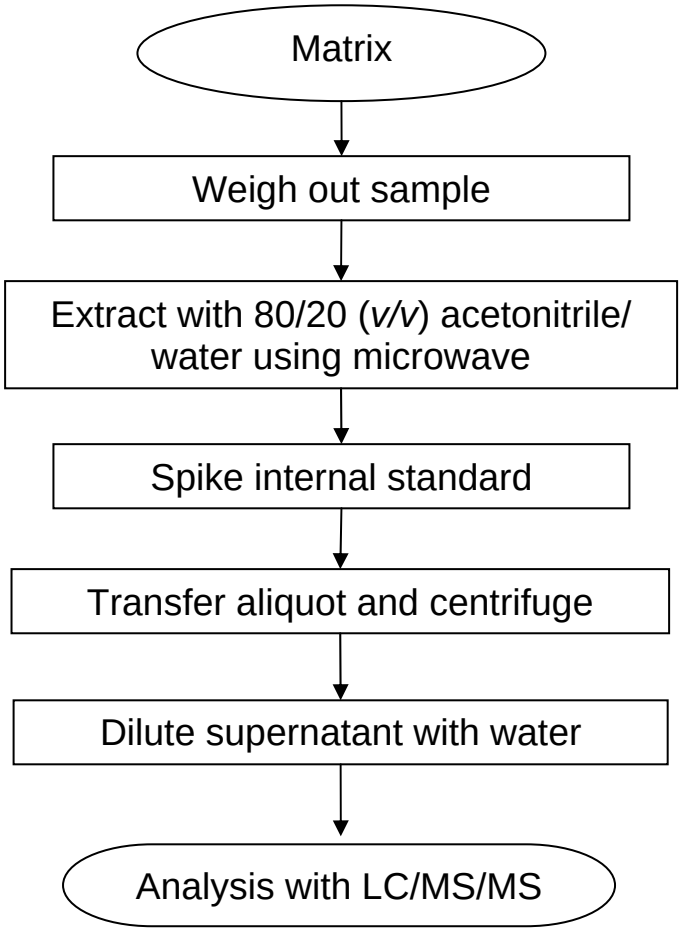
(Appendix 1 Continued)

The calculated MDL's and LOQ's for this method are presented below:

Matrix	Analyte	MDL, ng/g	LOQ, ng/g
Soil	AE1170437	0.2	0.8
	AE1170437 Diaminotriazine	0.1	0.3
	AE1170437 Hydroxyethyl	0.1	0.3
	AE1170437 Carboxylic Acid	0.1	0.4
	AE1170437 Triazine-indanone	0.1	0.3
	AE1170437 Olefin	0.1	0.5
Sediment	AE1170437	0.1	0.2
	AE1170437 Diaminotriazine	0.1	0.3
	AE1170437 Hydroxyethyl	0.1	0.4
	AE1170437 Carboxylic Acid	0.1	0.3
	AE1170437 Triazine-indanone	0.1	0.2
	AE1170437 Olefin	0.1	0.4

Note: The MDL calculated here is only to be considered an estimate. Each laboratory should evaluate its own MDL when reporting data. For practical reporting purposes, an LOQ of 1.5 ppb and an MDL of 0.3 ppb for all analytes is recommended to take into account variation between instruments over time.

Appendix 2 Method Flow Chart



Appendix 4 Instrument Conditions For AE1170437 and its metabolites

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

LC/MS/MS Parameters

NOTE: 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. Therefore, the given LC/MS/MS parameters listed below are guidelines and may be modified. These parameters should be optimized for the instrument and column actually used. Also, instrument parameters and mobile phase may be adjusted to improve separation from any observed interfering peaks.

The following abbreviations are used in the LC/MS/MS acquisition parameters listed below

MRM	Multiple Reaction Monitoring
MCA	Multiple Channel Acquisition
DP	Declustering Potential
EP	Entrance Potential
CE	Collision Energy
CXP	Collision Cell Exit Potential
CAD:	Collision gas (Collision Activated Dissociation)
CUR:	Curtain gas
GS1:	Ion Source Gas 1
GS2:	Ion Source Gas 2
IS:	Ion Spray Voltage
TEM:	Temperature
ihe:	Interface Heater
CEM	Channel Electron Multiplier
DF	Deflector

Log Information from Devices at Start of acquisition:

Integrated System	Shimadzu Controller	SCL10Avp
Pressure Units	psi	
AutoSampler	PE200	
Loop Volume (given by user)	100 µl	
Injection Volume used	40 µl.	
Pump	Shimadzu LC10ADvp	
Pump	Shimadzu LC10AD	
Mass Spectrometer	API 4000	
Firmware Version	M401402 B4T0301 M3L1415 B3T0300	
Component Name	Triple Quadrupole LC/MS/MS Mass Spectrometer	
Component ID	API 4000	
Manufacturer	AB Sciex Instruments	
Model	1005760-AA	
Serial Number	V0110920603	

(Appendix 4 Continued)

Acquisition Info

Sample Acq Duration:	13min60sec
Number of Scans:	4828
Periods in File:	6
Software Version:	Analyst 1.4.1

Shimadzu LC Method Properties

Shimadzu LC system Equlibration time = 0.00 min
Shimadzu LC Method Parameters
Pumps
=====
Pump A Model: LC-10ADvp
Pump C Model: LC-10AD
Binary Gradient Total Flow: 0.200 mL/min.
Pump C Pct: 40.0
Pressure Range: 0 - 5000 psi
System Controller
==========
Model: SCL-10Avp
Power: On
Event 1: Off
Event 2: Off
Event 3: Off
Event 4: Off

Time Program
==========

Time	Module	Events	Parameter
0.01	Pumps	%C	40
0.20	Pumps	%C	40
1.00	Pumps	%C	65
4.00	Pumps	%C	70
6.00	Pumps	%C	80
6.50	Pumps	%C	95
9.90	Pumps	%C	95
10.00	Pumps	%C	40
14.00	System Controller	Stop	

PE200 Autosampler Properties

Inject Details	
Syringe Size (µl):	250
Injection Volume (µl):	40
Flush Details	
Pre-inject Flushes (#):	0
Post-inject Flushes (#):	6
Inject Details (Advanced)	
Air Cushion (µl):	10

(Appendix 4 Continued)

Excess Volume (µl):	10
Sample Speed:	Medium
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):	100
Flush Details (Advanced)	

Flush Volume (µl):	500
Flush Speed:	Slow
Temperature Control:	Disable

Quantitation Information:

Sample Type:	Unknown
Dilution Factor:	1.000000

Period: 1

Period 1 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Thres.:	0.00 cps
Settling Time:	700.0000 msec
MR Pause:	5.0070 msec

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
500.00	400.00	100.00

Parameter Table(Period 1 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	50.00
GS2:	50.00
TEM:	500.00
ihe:	ON
IS:	-4500.00
DP	-101.00
EP	-10.00
CE	-21.00
CXP	-12.00

(Appendix 4 Continued)

Period 2: (Period for AE1170437 Diaminotriazine)

Scans in Period:	327
Relative Start Time:	4.00 min
Experiments in Period:	1

Period 2 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
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

AE1170437 Diaminotriazine (Retention time ~4.9 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
158.00	138.00	200.00
Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
165.00	145.00	100.00

Parameter Table (Period 2 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	50.00
GS2:	50.00
TEM:	500.00
ihe:	ON
IS:	5500.00
DP	56.00
EP	10.00
CE	21.00
CXP	10.00

Period 3: (Period for AE1170437 Hydroxyethyl)

Scans in Period:	348
Relative Start Time:	5.70 min
Experiments in Period:	1

Period 3 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Low
Intensity Thres.:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0070 msec

(Appendix 4 Continued)

AE1170437 Hydroxyethyl (Retention time ~6.2 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
300.00	156.00	200.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
304.00	160.00	100.00

Parameter Table (Period 3 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	50.00
GS2:	50.00
TEM:	500.00
ihe:	ON
IS:	5500.00
DP	86.00
EP	10.00
CE	25.00
CXP	12.00

Period 4: (Period for AE1170437 Carboxylic Acid, AE1170437 Triazine-indanone, AE1170437 Olefin)

Scans in Period:	161
Relative Start Time:	7.50 min
Experiments in Period:	1

Period 4 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Low
Intensity Thres.:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0070 msec

AE1170437 Carboxylic Acid (Retention time ~8.2 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
332.00	158.00	200.00	DP	92.00
			CE	29.00
			CXP	16.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
336.00	162.00	100.00	DP	92.00
			CE	29.00
			CXP	16.00

(Appendix 4 Continued)

AE1170437 Triazine-indanone (Retention time ~8.4 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
316.00	158.00	200.00	DP	80.00
			CE	30.00
			CXP	11.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
320.00	162.00	100.00	DP	80.00
			CE	30.00
			CXP	11.00

AE1170437 Olefin (Retention time ~9.0 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
282.00	138.00	200.00	DP	93.00
			CE	23.00
			CXP	14.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)	Param	V
286.00	142.00	100.00	DP	93.00
			CE	23.00
			CXP	4.00

Parameter Table(Period 4 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	30.00
GS2:	10.00
TEM:	500.00
ihe:	ON
IS:	5500.00
EP	10.00

Period 5: (Period for AE1170437)

Scans in Period:	290
Relative Start Time:	10.00 min
Experiments in Period:	1

Period 5 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Positive
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Low
Intensity Thres.:	0.00 cps
Settling Time:	0.0000 msec
MR Pause:	5.0070 msec

(Appendix 4 Continued)

AE1170437 (Retention time ~10.5 min)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
302.00	158.00	200.00

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
306.00	162.00	100.00

Parameter Table(Period 5 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	50.00
GS2:	50.00
TEM:	500.00
ihe:	ON
IS:	5500.00
DP	61.00
EP	10.00
CE	17.00
CXP	15.00

Period 6 Experiment 1:

Scan Type:	MRM (MRM)
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
Intensity Thres.:	0.00 cps
Settling Time:	700.0000 msec
MR Pause:	5.0070 msec

Q1 Mass (amu)	Q3 Mass (amu)	Dwell(msec)
500.00	400.00	100.00

Parameter Table(Period 6 Experiment 1)

CAD:	12.00
CUR:	30.00
GS1:	50.00
GS2:	50.00
TEM:	500.00
ihe:	ON
IS:	-4500.00
DP	-120.00
EP	-10.00
CE	-52.00
CXP	-13.00

(Appendix 4 Continued)

Instrument Parameters:
Detector Parameters (Positive):

CEM	2200.0
DF	-100.0

Detector Parameters (Negative):

CEM	2200.0
DF	350.0

Keyed Text:
File was created with the software version: Analyst 1.4.1

Appendix 6 Example Calculation

An example calculation for AE1170437 from sample LOQ8 (California soil bulk control with LOQ spike), which was analyzed during the method validation study is presented below. This sample was fortified with 1.5 ppb AE1170437 and its metabolites AE1170437 Carboxylic Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201) and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine). The chromatogram used in this example is presented in Appendix 5 (Chromatogram 5) and the calibration curve for this analysis is presented in Appendix 3.

The standards were fit to the linear equation: $y = Ax + B$

Where: x is the concentration of the reference standard in ng/mL
A is the calibration line slope
B is the calibration line intercept (B is zero if the calibration line is through zero)
y is the native peak area/isotopic peak area ratio

The example shown below is for the calculation of AE1170437 residues. AE1170437 Carboxylic Acid, AE1170437 Triazine-indanone, AE1170437 Hydroxyethyl, AE1170437 Olefin, and AE1170437 Diaminotriazine residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in parts per billion was determined. The concentration in parts per billion (ppb) of AE1170437 in the soil was calculated using the following equation,

$$E = \frac{(y - B)}{A} \times D$$

Where: E = concentration of AE1170437 in sample (ppb or ng/g)
D = dilution factor (e.g. 20 mL/g in this method)

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

W	V_1	V_2	V_3	Native Peak Area	IS Peak Area	y	A	B
15 g	30 mL	0.1 mL	1 mL	56391	236890	0.2380	2.923*	0

* Data is observed from AE1170437 Calibration Curve in Appendix 3.

From the above equations:

$$\text{Dilution Factor } D = \frac{30\ mL}{15\ g} \times \frac{1\ mL}{0.1\ mL} = 20\ mL/g$$

$$AE117437\ \ Found = \frac{(0.2380 - 0)}{2.923} \times 20 = 1.63\ ng/g\ (ppb)$$

Therefore this sample contains 1.63 ppb AE1170437.

(Appendix 6 Continued)

The % recovery was calculated 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

Therefore, for this sample, which was fortified with 1.5 ppb AE1170437:

R = 1.63 ppb
S = 0.0 ppb
T = 1.5 ppb

$$Recovery\ of\ AE1170437\ (\%) = \frac{(1.63 - 0)}{1.5} \times 100\% = 109\%$$

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

Appendix 7 Revision History

Method #	Revision	Description
DH-002-S06-01	01	Method prepared on completion of validation study
DH-002-S06-02	02	Method is updated with the result from ILV