1. SUMMARY

This method is suitable for the determination of the 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 water including surface water, groundwater, and drinking water.

An aliquot of water containing AE1170437 and its metabolites is fortified with isotopically labeled internal standards of AE1170437 and its metabolites. The sample is directly injected to liquid chromatograph-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.

The data generated during the method validation study[1] indicate that the limit of quantitation (LOQ) lies at or below the target LOQ of 0.05 ng/mL (ppb) for AE1170437 and its metabolites AE1170437 carboxylic acid, AE1170437 triazine-indanone, AE1170437 hydroxyethyl, AE1170437 olefin, and AE1170437 diaminotriazine. For practical reporting purposes, an LOQ of 0.05 ppb and a Method Detection Limit (MDL) of 0.02 ppb for all analytes is recommended to allow for variation over time and between instruments.

The mean recovery and relative standard deviation (RSD) determined for AE1170437 and its metabolites based on multiple fortifications at 0.05 ppb (LOQ) and 0.5 ppb (10X LOQ) were all within the range of 70 to 110%, and the precision values as measured by the RSD were all less than 20%.

2. BACKGROUND

The non-selective residual herbicide AE1170437 is currently being developed by Bayer CropScience. During the conduct of this study, the technical grade active substance mixture of AE1170437 and its isomer AE1170438 (0% to 5.3%) have been assigned a new internal Bayer CropScience code of BCS AA10717. But for consistency, AE1170437 is still used in this report.

An analytical method was developed for the analysis 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 water. The structures for these compounds are presented in Section 4.

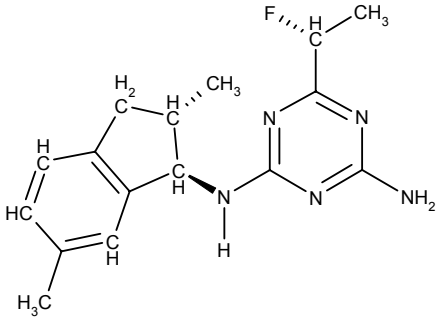
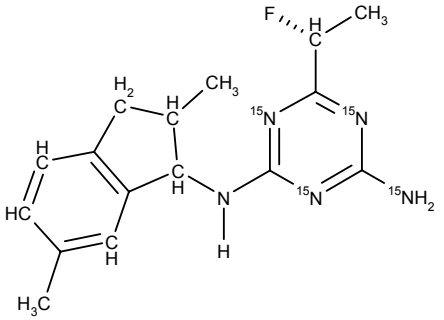
The method was validated in Bayer CropScience Study Number RADHP043[1]. This method report was prepared based on the results obtained in the validation study.

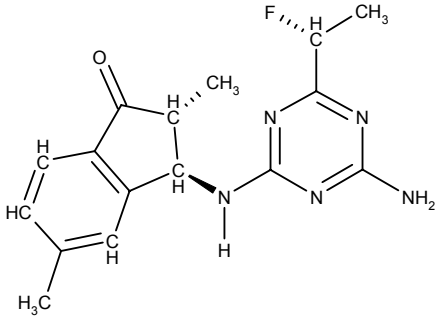
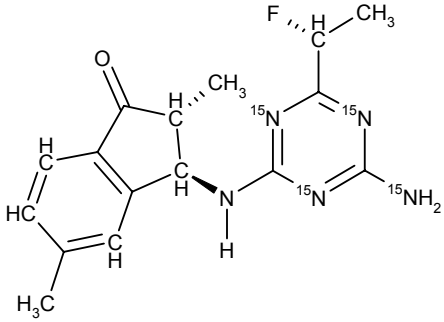
3. PRINCIPLE

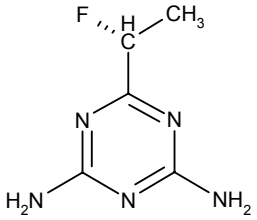
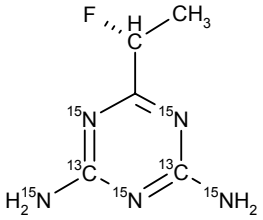
An aliquot of water is fortified with isotopically labeled internal standards of AE1170437 and its metabolites. The sample is directly injected to liquid chromatograph-tandem mass spectrometry (LC/MS/MS). Quantification is based on the use of internal standards and comparison of peak areas with those of known standards.

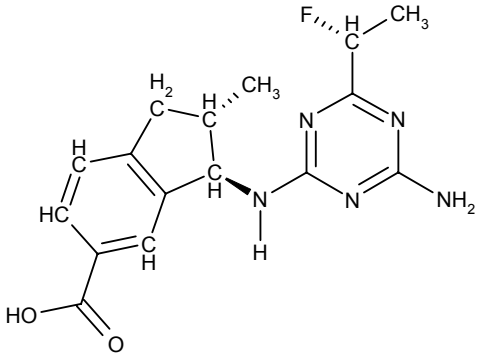
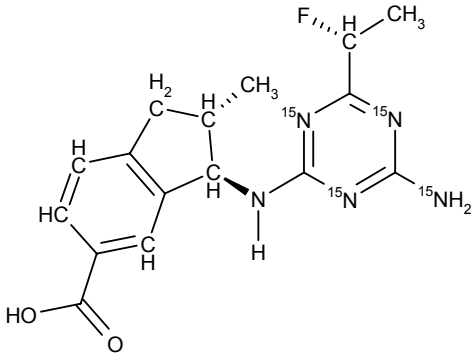
4. COMPOUNDS

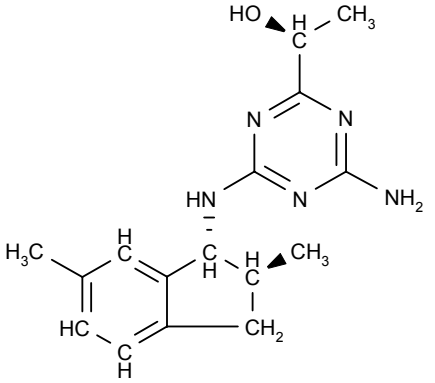
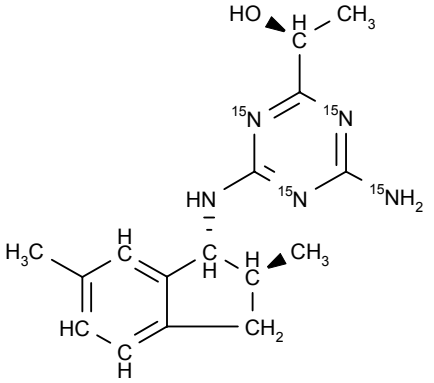
The structures for AE1170437, its metabolites and their associated internal standards are presented below:

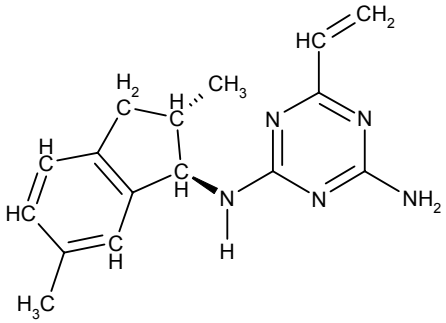
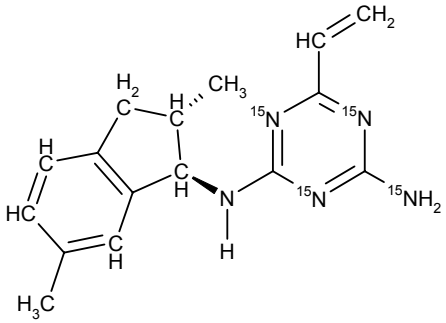
Code Name:	AE1170437 (Parent)	AE1170437-isomer mix-triazine-$^{15}N_4$ (Internal standard for AE1170437)
Structure		
Chemical Name:	N-[(1R,2S)-2,3-Dihydro-2,6-dimethyl-1H-inden-1-yl]-6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine	N-[(1R,2S)-2,3-Dihydro-2,6-dimethyl-1H-inden-1-yl]-6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine-$^{15}N_4$
Molecular Weight:	301.4, Monoisotopic mass: 301.1703	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	*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-$^{15}N_4$
Molecular Weight:	315.3, Monoisotopic mass: 315.1495	319.3
Molecular Formula:	$C_{16}H_{18}FN_5O$	$C_{16}H_{18}F^{15}N_4N$ O

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	6-[(1*R*)-1-Fluoroethyl]-1,3,5-triazine-2,4-diamine-$^{15}N_5$, $^{13}C_2$
Molecular Weight:	157.1, Monoisotopic mass: 157.0763	164.1
Molecular Formula:	$C_5H_8FN_5$	$C_5H_8F^{15}N_5{}^{13}C_2$

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	(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-$^{15}N_4$
Molecular Weight:	331.3, Monoisotopic mass: 331.1444	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	(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-$^{15}N_4$
Molecular Weight:	299.4, Monoisotopic mass: 299.1746	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	N-[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]-6-vinyl-1,3,5-triazine-2,4-diamine-$^{15}N_4$
Molecular Weight:	281.4, Monoisotopic mass: 281.1640	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
- Disposable pipettes
- Glass “Class A” graduated cylinders, pipettes, Gastight® micro-syringe, and volumetric flasks
- Micropipette, Eppendorf with disposable tips or equivalent
- Nalgene® HDPE Narrow-Mouth Sample Bottles 125 mL, (Fisher Scientific, Cat. No. 03-313-1C)
- 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)
- Sodium hypochlorite, 13% active chlorine (Fisher Scientific Cat. No. AC21925-0025)
- Sodium thiosulfate, Crystal $Na_2S_2O_3 \cdot 5H_2O$, 100.3% (Mallinckrodt Cat. No. 8100 KLPG)
- 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. SOLUTIONS

0.05% Formic Acid in HPLC Grade Water (or Methanol) (for use as a mobile phase component)

Add about 900 mL of HPLC grade water (or methanol) into a 1000-mL graduated cylinder.

Transfer 0.5 mL of formic acid to that cylinder and dilute to the 1000-mL mark with HPLC grade water (or methanol).

If necessary, transfer the solution to a clean, dry mobile phase reservoir.

Swirl to mix thoroughly, but do not shake, minimizing the amount of air dissolved in the solution. If necessary, place the container or reservoir in a sonicator bath and apply vacuum while sonicating for about 10 minutes until air bubble formation or cavitation subsides to a minimum. Alternatively, use an in-line degasser.

Solution of 3 mg/mL sodium thiosulfate

Weigh approximately 470 mg of sodium thiosulfate into a 100 mL volumetric flask. Dissolve the amount in approximately 50 mL of HPLC grade water and dilute to the 100 mL mark with HPLC water. Mix thoroughly by inverting the flask several times. This solution is 3 mg/mL or 3000 ppm. Transfer the sodium thiosulfate solution to a 100 mL amber bottle and store refrigerated at ~ 4 ºC.

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. Dilute to volume with 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 1 mL of the 200 µg/mL free chlorine solution to a 100 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 ~ 4 ºC and should be remade if more than three weeks old.

8. 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.

8.1 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 may vary among the analytes.

8.2 Fortification Standard Solutions

1. Native mixed working solution (500 ng/mL): Transfer a 500 µL aliquot of each of the 100,000 ng/mL native AE1170437 and metabolite stock solutions (Section 8.1) into a 100 mL volumetric flask. Dilute to 100 mL with ACN. The concentration of this solution is 500 ng/mL for each analyte.

2. 10XLOQ mixed spike solution (50 ng/mL): Transfer a 50 µL aliquot of each of the 100,000 ng/mL native AE1170437 and metabolite stock solutions (Section 8.1) into a 100 mL volumetric flask. Dilute to 100 mL with ACN. The concentration of this solution is 50 ng/mL for each analyte. A 10 mL water sample is fortified to 0.5 ng/mL (10XLOQ) by the addition of 100 µL of this 50 ng/mL solution.

3. LOQ mixed spike solution (5 ng/mL): Transfer a 1000 µL aliquot of native working solution (500 ng/mL) (step 1) into a 100 mL volumetric flask. Dilute to 100 mL with ACN. The concentration of this solution is 5 ng/mL for each analyte. A 10 mL water sample is fortified to 0.05 ng/mL (LOQ) by the addition of 100 µL of this 5 ng/mL solution.

4. Internal standard (IS) mixed spike solution: Transfer a 25 µL aliquot of each of the 100,000 ng/mL isotopically labeled AE1170437 and metabolite stock solutions (Section 8.1) into a 100 mL volumetric flask. Dilute to 100 mL with ACN. The concentration of this solution is 25 ng/mL for each analyte. A 10 mL water sample is fortified to 0.25 ng/mL by the addition of 100 µL of this 25 ng/mL solution.

Name of Standard Solutions	Concentration of Solution Used for Dilution (ng/mL)	Aliquot Taken (µL)	Dilution Volume (mL)	Concentration of New Solution (ng/mL)
Native Working Solution	100,000 (individual stock solution)	500	100	500 (native mixed)
10X LOQ Spike Solution		50		50 (native mixed)
IS Spike Solution		25		25 (IS mixed)
LOQ Spike Solution	500 (native mix)	1000	100	5 (native mix)

8.3 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 1.0 ng/mL of native analytes and 0.25 ng/mL of isotopically labeled analytes (last solution in the table below), take 200 µL of the 500 ng/mL mixed native standard solution (Section 8.2.2) and place it in a 100-mL volumetric flask. Take 1000 µL of the 25 ng/mL mixed isotopically labeled internal standard solution (Section 8.2.3) and add to the same volumetric flask. Bring volume to the mark with water. Cap the 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	50 25	0 1000	100	0.00 0.25
Mixed Native Mixed IS	50 25	50 1000	100	0.025 0.25
Mixed Native Mixed IS	50 25	100 1000	100	0.05 0.25
Mixed Native Mixed IS	50 25	200 1000	100	0.1 0.25
Mixed Native Mixed IS	500 25	100 1000	100	0.5 0.25
Mixed Native Mixed IS	500 25	200 1000	100	1.0 0.25

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 1.

9. ANALYTICAL PROCEDURE FOR ANALYSIS OF WATER

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

9.1 Laboratory Fortified Sample Preparation

Sample fortification is performed by adding a certain amount of a fortification solution to 10 mL of water to bring a desired fortification level. For example, fortification at the LOQ of 0.05 ng/mL would involve addition of 100 µL of 5 ng/mL mixed native fortification solution (Section 8.2.3) to 10 mL water. Thoroughly mix the sample by inversion.

9.2 Analytical Procedure

In order to reduce the potential degradation of target compounds by free chlorine contained in drinking water, sodium thoisulfate should be added to the sample bottle of drinking water before sample collection. 15 ppm of sodium thiosulfate added to the water sample is sufficient to remove up to about 2 ppm of chlorine and stabilize any AE 1170437 and its metabolite residues present.

1. Pipet a 10-mL aliquot of UTC water into each of two separate 20-mL vials. When analyzing drinking water, add 50 µL of 3,000 ppm sodium thiosulfate solution to each vial before pipetting the aliquot of water into the vial, and mix by inverting several times after adding the aliquot.

2. To one of the UTC water samples add an appropriate volume of fortification solution. For example, add 100 µL of a 5 ng/mL fortification solution to a 10 mL sample or 100 µL of a 50 ng/mL fortification solution to a 10 mL sample to give approximately 0.050 ppb (LOQ) and 0.500 ppb (10XLOQ) analyte concentrations, respectively. The other UTC sample is used as a blank control.
3. Pipet a 10-mL aliquot of each sample to be analyzed into separate 20 mL vials.

4. Add 100 µL of the 25 ng/mL labeled internal standard to the UTC, the fortified UTC and each sample. Cap the vials and mix by inverting several times.

5. Transfer an aliquot of each sample to an HPLC vial, the samples are ready for LC/MS/MS analysis.

Note: If a sample is too concentrated in any analyte for the calibration curves used, either add a standard of higher concentration to the calibration curve or dilute the sample with water before adding the internal standard. This ensures that the internal standard level in the sample is the same as in the calibration standards. Filter the surface water sample with 0.45 µm GF/F filter before LC/MS/MS analysis, if necessary.

10. LC/MC/MC ANALYSIS

10.1 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)

10.2 Compound Identification

The analytes are identified by comparing the acquired mass spectra and retention times for each sample to the reference spectra and retention times for the calibration standards acquired under the 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	3.9	158	138	56	21	10
AE1170437 Diaminotriazine-$^{15}N_5$,$^{13}C_2$	3.9	165	145	56	21	10
AE1170437 Hydroxyethyl	5.9	300	156	86	25	12
AE1170437 Hydroxyethyl-$^{15}N_4$	5.9	304	160	86	25	12
AE1170437 Carboxylic Acid	8.1	332	158	92	29	16
AE1170437 Carboxylic Acid-$^{15}N_4$	8.1	336	162	92	29	16
AE1170437 Triazine-indanone	8.3	316	158	80	30	11
AE1170437 Triazine-indanone-$^{15}N_4$	8.3	320	162	80	30	11
AE1170437 Olefin	8.8	282	138	93	23	14
AE1170437 Olefin-$^{15}N_4$	8.8	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

10.3 Sample Analysis

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

Inject a 40 µL aliquot of each test sample (or fortified sample matrix) in Section 10.2 onto the LC/MS/MS under the conditions presented in Appendix 3. 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 4.

10.4 LC/MS/MS Standard Calibration and Residue Calculations

The calculation displayed below is from the method validation. Alternate calculation procedures appropriate to the reporting requirements may be substituted. An example calculation is presented in Appendix 5.

Standardize the LC/MS/MS response under the conditions outlined in Appendix 3 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 should 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 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)

$$y = \text{Response ratio} = \frac{\text{Analyte Response Area}}{\text{IS Rponse Area}}$$

The equation to calculate the residues in the samples is:

$$C = \frac{y - B}{A}$$

Where: C = concentration of analyte in sample in parts per billion (ppb or ng/mL)
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)

10.5 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 0.05 ppb in water or other appropriate level with fortification solutions. Calculate the final residue R for the control (S) and fortified control (R) samples.

11.3 Confirmatory Method

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

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: 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

11.4 Time Considerations

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

12. 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.

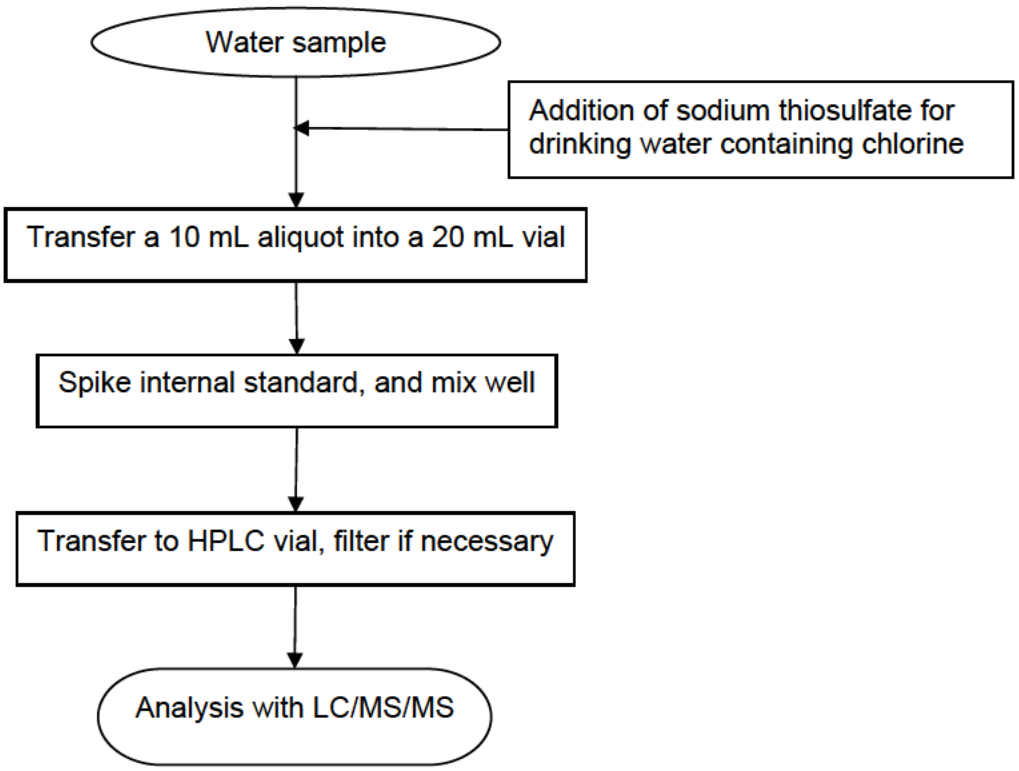
13. REFERENCES

1. T Xu, In House Laboratory Validation of an Analytical Method for the Determination of Residues of AE1170437 and its metabolites AE1170437 Acid (AE2158969), AE1170437 Triazine-indanone (AE2158968), AE1170437 Hydroxyethyl (AE2300077), AE1170437 Olefin (BCS-AA10201), and AE1170437 Diaminotriazine (1-Fluoroethyl triazinediamine) in Water Using LC/MS/MS, Bayer Study Number: RADHP043.
2. Krebber, R., Independent laboratory validation of method DH-005-W07-01 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 water using LC/MS/MS. Bayer CropScience AG, Monheim, Germany, Bayer Study Number: RADHP066,

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 Water		
Method ID	DH-005-W07-01	
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	Direct injection	
Cleanup Strategies	None	
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. 8.1 min. 8.3 min. 5.9 min. 8.8 min. 3.9 min.

Appendix 2 Method Flow Chart



Appendix 3 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
AutoSampler	PE200
Loop Volume (given by user)	100 µl
Injection Volume used	40 µl.
Pump	Shimadzu LC10ADvp
Pump	Shimadzu LC10AD
Mass Spectrometer	API 4000
Component Name	Triple Quadrupole LC/MS/MS Mass Spectrometer
Component ID	API 4000
Manufacturer	AB Sciex Instruments
Model	1005760-AA

Acquisition Info

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

(Appendix 3 Continued)

Shimadzu LC Method Properties

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

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

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)

(Appendix 3 Continued)

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

Quantitation Information:
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

Period 2: (Period for AE1170437 Diaminotriazine)

Scans in Period:	327
Relative Start Time:	3.2 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

(Appendix 3 Continued)

AE1170437 Diaminotriazine (Retention time ~3.8 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:	5.0 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

AE1170437 Hydroxyethyl (Retention time ~5.8 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

(Appendix 3 Continued)

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:	7.0 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 ~7.9 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

AE1170437 Triazine-indanone (Retention time ~8.1 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 ~8.4 min)

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

(Appendix 3 Continued)

			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:	9.60 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

AE1170437 (Retention time ~10.3 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

(Appendix 3 Continued)

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

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 5 Example Calculation

An example calculation for AE1170437 from sample 07DH-DW-LOQ1 (drinking water control with LOQ spike), which was analyzed during the method validation study is presented below. This sample was fortified with 0.05 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 4 (Chromatogram 5) and the calibration curve for this analysis is presented in Appendix 1.

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 water was calculated using the following equation,

$$C = \frac{y - B}{A}$$

Where: C = concentration of AE1170437 in sample (ppb or ng/mL)

Native Peak Area*	IS Peak Area*	y	A**	B**	C, ppb
57811.1	171000	0.3381	6.888	0	0.0491

*: Data is observed from Chromatogram 5 in Appendix 4.
**: Data is observed from AE1170437 Calibration Curve in Appendix 1.

From the above equations:

$$y = \text{Response ratio} = \frac{\text{Compound} \cdot \text{Response} \cdot \text{Area}}{\text{IS} \cdot \text{Rponse} \cdot \text{Area}} = \frac{57811.1}{171000} = 0.3381$$

$$\text{AE117437 Found} = \frac{(0.3381 - 0)}{6.888} = 0.0491 \text{ ng/mL (ppb)}$$

Therefore this sample contains 0.0491 ppb AE1170437.

(Appendix 5 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 0.05 ppb AE1170437:

R = 0.0491 ppb
S = 0.0 ppb
T = 0.05 ppb

$$\text{Recovery of AE1170437 } (\%) = \frac{(0.0491 - 0)}{0.05} \times 100\% = 98.2\%$$

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

(Appendix 6 Continued)

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

Analyte	MDL, ng/mL	LOQ, ng/mL
AE1170437	0.004	0.01
AE1170437 Diaminotriazine	0.005	0.02
AE1170437 Hydroxyethyl	0.003	0.01
AE1170437 Carboxylic Acid	0.003	0.01
AE1170437 Triazine-indanone	0.003	0.01
AE1170437 Olefin	0.006	0.02

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 0.05 ppb and an MDL of 0.02 ppb for all analytes is recommended to take into account variation between instruments over time.

Appendix 7 Revision History

Method #	Revision	Description
DH-005-W07-01	01	Method prepared on completion of validation study[1]
DH-005-W07-02	02	Updated with Independent Laboratory Validation (ILV)[2] result, and corrected calculated MDL and LOQ values in Appendix 6