2 Objective

The objective of this study was to demonstrate that method DH-005-W07-01 ("Method of Analysis 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") can be performed with acceptable recoveries for determination of the compound AE1170437 and its metabolites at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience Environmental Chemistry, Stilwell, USA, and reported as Method DH-005-W07-01, by Tianbo Xu, Ph.D., dated December 18, 2007. River Rhine water (taken at Leverkusen-Hitdorf, Germany) and tap water Monheim, Germany were chosen as representative matrices for validation within the present study.

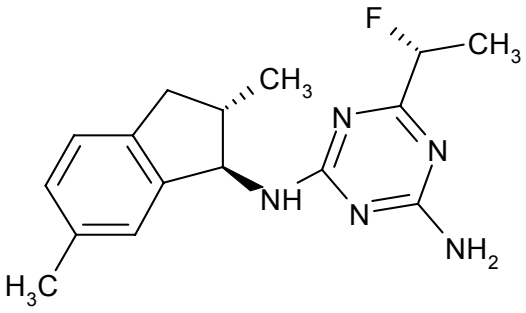
This study was performed in accordance with US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996.

3 Materials

3.1 Test and Reference Items

Code name:	**AE 1170437 (BCS-AA10717)**
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
Empirical formula:	C_{16} H_{20} F N_5
CAS Number	730979-19-8
Molecular weight:	301.4 g/mol

Structural formula:



Certificate of analysis:	AZ 14674
Origin Batch No.:	BCMF1467-53-3
Purity:	99.6%
Expiry date:	2010-12-18
Appearance:	off white powder

Code Name: **AE 1170437-isomer mix-triazine-$^{15}N_4$**

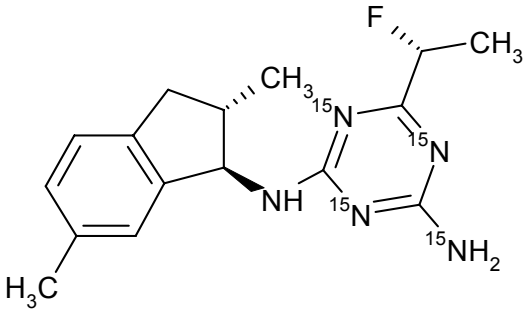
(Isotopic Internal Standard for AE1170437)

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-$^{15}N_4$

Molecular Formula: $C_{16}\ H_{20}\ F\ ^{15}N_4\ N$

Molecular Weight: 305.3 g/mol

Structural formula:



Certificate of analysis: K-1638
Reference ID: 2005BRP022-0031
Appearance: white solid

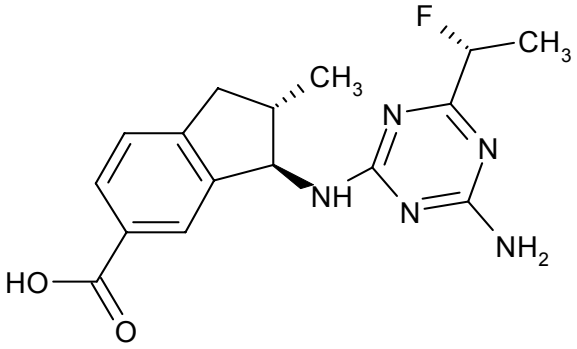
Code name: **AE 2158969 (AE 1170437 Carboxylic acid)**

Chemical Name: (2S,3R)-3-{4-Amino-6-[(1R)-1-fluoroethyl]-1,3,5-triazin-2-ylamino}-2-methylindane-5-carboxylic acid

Empirical formula: $C_{16}\ H_{18}\ F\ N_5\ O_2$

Molecular weight: 331.4 g/mol

Structural formula:



Certificate of analysis: AZ 13481
Batch ID: KATH4548-12-4
Purity: 98.2%
Expiry date: 2008-07-26
Appearance: off white powder

Code Name: **AE 1170437 Acid-triazine-$^{15}N_4$**

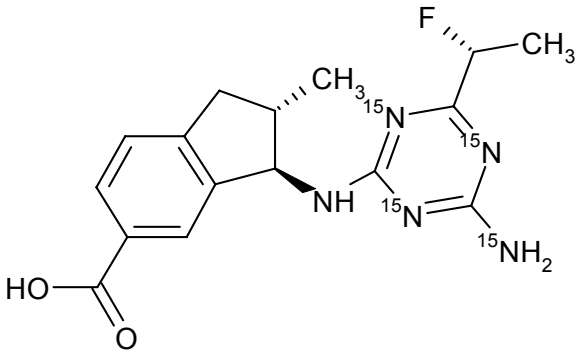
(Isotopic internal standard for AE1170437 carboxylic acid)

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-$^{15}N_4$

Molecular Formula: $C_{16}\ H_{18}\ F\ {}^{15}N_4\ N\ O_2$

Molecular Weight: 335.3 g/mol

Structural formula:



Certificate of analysis: K-1654
Reference ID: 2005BRP027-025
Appearance: white solid

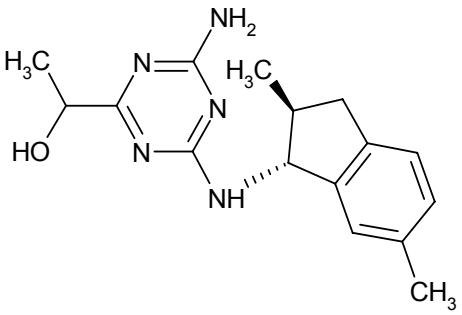
Code name: ***AE 22300077*** **(AE 1170437-Hydroxyethyl)**

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

Empirical formula: $C_{16}\ H_{21}\ N_5\ O$

Molecular weight: 299.4 g/mol

Structural formula:



Certificate of analysis: AZ 13753
Batch ID: KTS 10306-1-3
Purity: 99.0%
Expiry date: 2008-10-13

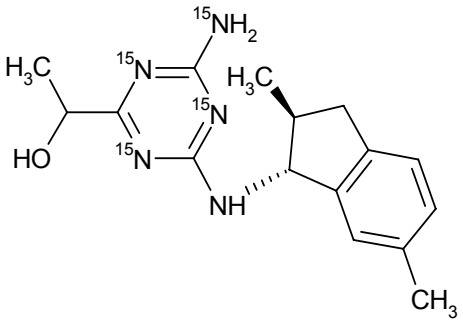
Code name: **AE 1170437 desflourohydroxy-triazine-$^{15}N_4$**
(Isotopic internal standard for AE1170437-hydroxyethyl)

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-$^{15}N_4$

Empirical formula: $C_{16}\ H_{21}\ ^{15}N_4\ N\ O$

Molecular weight: 303.3 g/mol

Structural formula:



Certificate of analysis: K-1650
Reference ID: 2005BRP022-0033
Purity: 97.6%
Expiry date: 2016-09-07

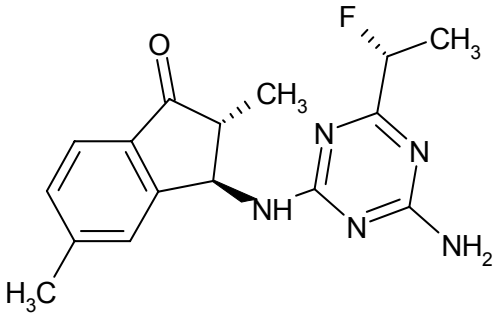
Code Name: **AE 2158968 (AE 1170437-Triazine-indanone)**

Chemical Name: (2R,3R)-3-{4-amino-6-[(1R)-1-flouroethyl]-1,3,5-triazin-2-ylamino}-2,5-dimethylindan-1-one

Molecular Formula: $C_{16}\ H_{18}\ F\ N_5\ O$

Molecular Weight: 315.3 g/mol

Structural formula:



Certificate of analysis: AZ 14135
Batch ID: KATH4545-8-11
Purity: 96.1%
Expiry date: 2009-03-26
Appearance: light yellow powder

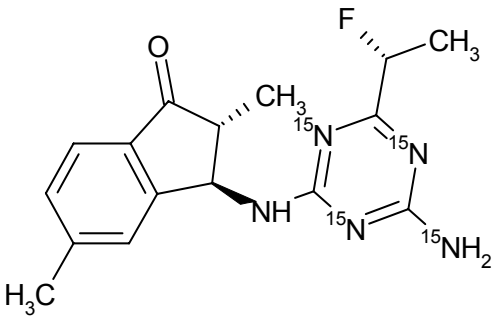
Code Name: **AE 1170437 ketone-triazine-$^{15}N_4$**
(Isotopic internal standard for AE1170437-triazine-indanone)

Chemical Name: N-[(1R,2S)-2,3-Dihydro-2,6-dimethyl-3-oxo-1H-inden-1-yl]-6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine-$^{15}N_4$

Molecular Formula: $C_{16}\ H_{18}\ F\ ^{15}N_4\ N\ O$

Molecular Weight: 319.3 g/mol

Structural formula:



Certificate of analysis: K-1639
Reference ID: 2005BRP022-0032
Purity: 94.9%
Expiry date: 2016-07-25

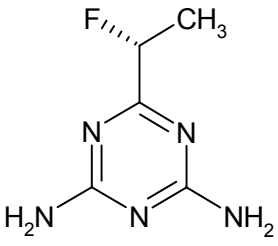
Code Name: **AE 1170437-Diaminotriazine (1-Fluoroethyl triazinediamine)**

Chemical Name: 6-[(1R)-1-Fluoroethyl]-1,3,5-triazine-2,4-diamine

Molecular Formula: $C_5\ H_8\ F\ N_5$

Molecular Weight: 157.1 g/mol

Structural formula:



Certificate of analysis: AZ 14207
Batch ID: GXA38-4-1+GXA38-3-1
Purity: 96.4%
Expiry date: 2010-06-11
Appearance: white powder

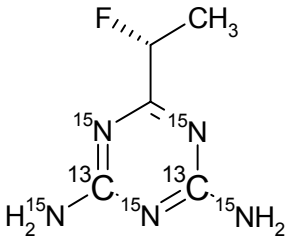
Code Name: **1-Fluoroethyl triazinediamine-$^{15}N_5$, $^{13}C_2$**
(Isotopic internal standard for AE1170437-diaminotriazine)

Chemical Name: 6-[(1R)-1-Fluoroethyl]-1,3,5-triazine-2,4-diamine-$^{15}N_5$, $^{13}C_2$

Molecular Formula: $C_3\ H_8\ F\ ^{15}N_5,\ ^{13}C_2$

Molecular Weight: 164.1 g/mol

Structural formula:



Certificate of analysis: K-1627
Reference ID: 2005BRP022-0026
Purity: 97.4%
Expiry date: 2016-05-22

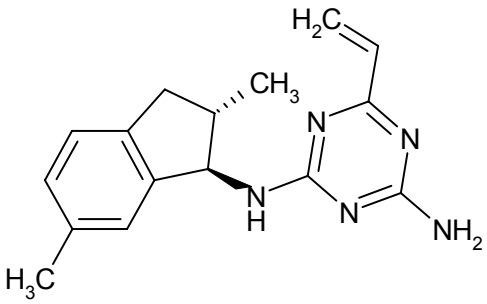
Code name: **BCS-AA10201 (AE 1170437-Olefin)**

Chemical Name: N-[(1R,2S)-2,6-Dimethyl-2,3-dihydro-1H-inden-1-yl]-6-vinyl-1,3,5-triazine-2,4-diamine

Empirical formula: $C_{16}\ H_{19}\ N_5$

Molecular weight: 281.4 g/mol

Structural formula:



Certificate of analysis: AZ 13730
Purity: 96%
Expiry date: 2008-10-12
Appearance: off white powder

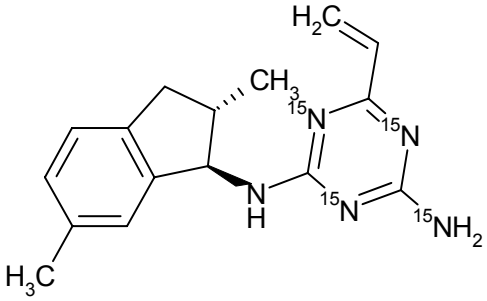
Code name: **BCS-AA10201-triazine-$^{15}N_4$**
(Isotopic internal standard for AE1170437-olefin)

Chemical Name: N-[(1R,2S)-2,6-Dimethyl-2,3-dihydro-1H-inden-1-yl]-6-vinyl-1,3,5-triazine-2,4-diamine -$^{15}N_4$

Empirical formula: $C_{16}\ H_{19}\ ^{15}N_4\ N$

Molecular weight: 285.3 g/mol

Structural formula:



Certificate of analysis: K-1651
Purity: 97.5%
Expiry date: 2016-09-20
Appearance: white solid

3.2 Test System

The method was validated using River Rhine and tap water Monheim. Two different water types were used in order to assess a possible influence of different water characteristics. The water samples were analysed for TOC, DOC, conductivity, water hardness, dry residue after filtration and pH by Bayer Industry Services, BIS SUA-PUA1, Building W15. Water types are summarised in Table 1.
Complete water parameters are reported in Table 13 and Table 14.

Table 1: Water Types

Water Type	Source of Water
Surface Water	River Rhine Water
Tap Water	Tap Water Monheim

4 Experimental

4.1 Analytical Method

The recovery data for the study were generated using the following method, which gives full details of preparing the analytical sample extracts and the conditions for high performance liquid chromatography (HPLC):

Number of the method:	DH-005-W07-01
Title of the method:	Method of Analysis 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
Author of the method:	Tianbo Xu, PH.D. Bayer CropScience Environmental Chemistry 17745 South Metcalf Avenue Stilwell, Kansas 66085
Reference:	Method DH-005-W07-01
Limit of quantitation:	0.05 µg/L

The following sample sets were analysed:

Table 2: Level and Number of Recoveries per Fortification Level

Water	Control sample	Level 0.5 µg/L	Level 5 µg/L
River Rhine	2	5	5
Tap Water Monheim	2	5	5

4.1.1 Outline of the Method

Appropriate volumes of fortification and internal standard solutions were added to the water samples. After mixing the analysis was performed by LC-MS/MS.

4.1.2 Instruments

Liquid Chromatograph:	HP 1100 Column Compartment G1316A HP 1100 Binary Pump G1312A HP 1100 Isocratic Pump G1310A HP 1100 Degasser G1322A Agilent, 40880 Ratingen, Germany
Autosampler:	HTC PAL System CTC Analytics AG 4222 Zwingen, Switzerland

Mass Spectrometer:	Applies Biosystems API 4000 with turbo-ionspray interface mass selective detector (MS/MS) Note: Some mass spectrometric conditions are instrument specific. The spectrometric conditions were optimised by a competent operator prior to analysis.

4.1.3 Reagents and Equipment

	Column (HPLC): Synergy Fusion-RP, length 250x2 mm, Particle size 4µm, pore size 80A Series No.: 00G-4424_B0, Phenomenex, 63741 Aschaffenburg, Germany
Methanol:	for HPLC, optigrade LGC Promochem GmbH, UN 1230 46485 Wesel, Germany
Formic acid (98-100%):	p.a. Riedel-de Haên, No.27001 64271 Darmstadt, Germany
Deionized water:	purified in a Milli-Q unit Milli-Pore GmbH 65731 Eschborn, Germany

Volumetric flasks, pipettes and other equipment commonly used in the laboratory.

4.1.4 Liquid Chromatographic Conditions

Liquid chromatographic conditions were as described in the original method report DH-005-W07-01.

Table 3: Liquid Chromatographic Conditions

Column:	Synergy Fusion-RP, pore size 80A length 250x2 mm
Particle size:	4 µm
Oven temperature:	25 °C
Injection volume:	100 µL
Run time:	14 minutes
Mobile phase:	A: deionized water (+ 0.05% formic acid) B: methanol (+ 0.05% formic acid)
Retention times:	approx. 10.9 min for AE1170437 approx. 8.8 min AE1170437 carboxylic acid (AE2158969) approx. 9.0 min AE1170437 triazine-indanone (AE2158968) approx. 6.9 min AE1170437 hydroxyethyl (AE2300077) approx. 9.5 min AE117043-Olefin (BCS-AA10201) approx. 4.4 min AE 1170437-Diaminotriazine

Table 4: HPLC Gradient

Time [min]	Flow [µL/min]	A [%]	B [%]
0.00	200	60.0	40.0
0.20	200	60.0	40.0
1.00	200	35.0	65.0
4.00	200	30.0	70.0
6.00	200	20.0	80.0
6.50	200	5.0	95.0
9.90	200	5.0	95.0
10.00	200	60.0	40.0
14.00	200	60.0	40.0

Table 5: Valco Valve Method Properties

Time [min]	
0.0	switch eluent stream into waste
1.0	switch eluent stream into interface
12.0	switch eluent stream into waste

These switching times were also adjusted to the used equipment.

4.1.5 Mass Spectrometric Parameters

MS/MS parameter settings were in general as described in method DH-005-W07-01 but optimized for the instrument being used.

4.1.6 Calculation

For calculation of the concentrations, five-point calibration curves were used. These curves were calculated using linear regression automatically after each sequence run with the Perkin-Elmer quantitation software Analyst (vers. 1.4.1). Further calculations were performed using the software MS-EXCEL 2002. The results given are rounded values. Thus, rounding "errors" may occur if recalculations are made using the listed figures.

4.1.6.1 Calculation of Analyte Concentrations

NOTE: Evaluation is performed according to the linearity standard procedure.

1. Calculate the response factors (peak area of detected analyte / peak area of the internal standard) of all standard injections and calculate the resulting linearity of the analyte.
2. Determine the response factor (peak area of detected analyte / peak area of the internal standard) for the sample. This value will be used as x.
3. Calculate the residue level in µg/L as follows:

$$R = (x - b) / a$$

R: Determined concentration of analyte in µg/L
x: Response factor of the sample
b: Interception from linear regression
a: Slope from linear regression

4.1.6.2 Calculation of Recoveries

1. Calculate the concentration in the recovery sample according to 4.1.6.1.
2. Calculate the percent recovery as follows:

$$\text{Recovery} = \frac{\text{Concentration } x \text{ } 100}{\text{Fortification Level}}$$

Recovery: Recovered concentration of analyte in % found in the fortified sample
Concentration: Concentration in the fortified sample in µg/L determined according to 4.1.6.1
Fortification level: Fortified concentration of analyte in µg/L