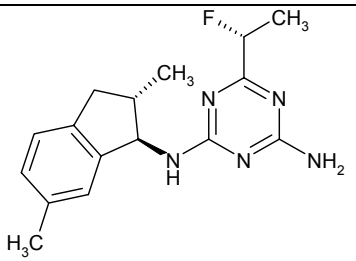
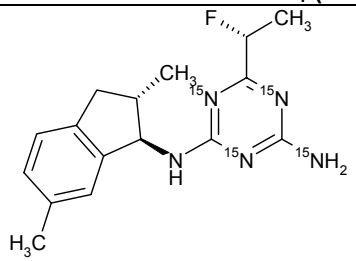
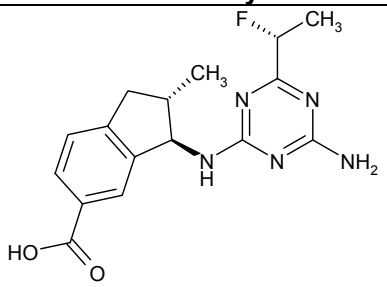
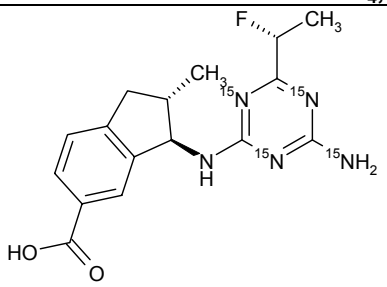
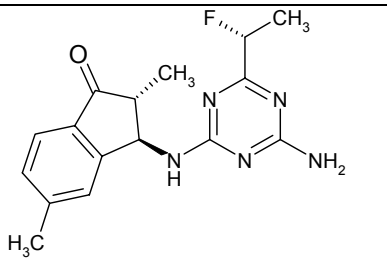
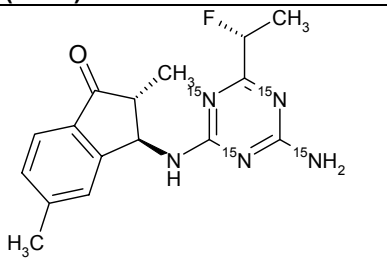
3 Materials

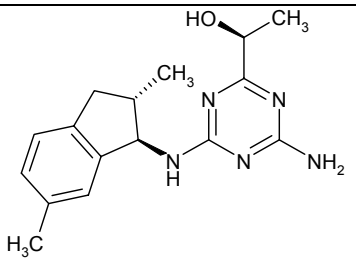
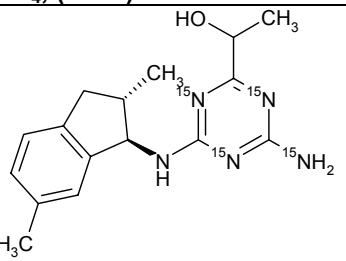
3.1 Test and Reference Items

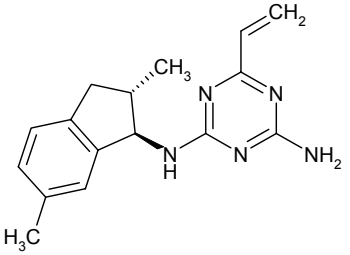
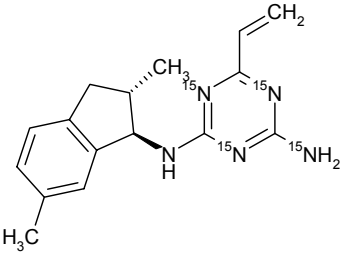
Test/reference Items and internal standards (ISTD's):

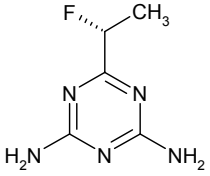
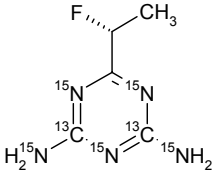
report/standard name	**AE1170437**	**AE1170437-triazine-$^{15}N_4$ (ISTD)**
		
IUPAC-name	N-[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]-6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine	
Formula:	$C_{16}H_{20}FN_5$	
MW, mean [g/mol]:	301.37	305.34
Certificate of analysis:	AZ 12604	K-1638
Purity:	99.4 %	99.5 %
Expiry date:	June 2007	
Origin:	Bayer CropScience GmbH, PT–Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

report/standard name	**AE1170437 Carboxylic Acid**	**AE1170437 Acid -triazine-$^{15}N_4$, (ISTD)**
		
IUPAC-name	(2S,3R)-3-({4-amino-6-[(1R)-1-fluoroethyl]-1,3,5-triazin-2-yl}amino)-2-methylindane-5-carboxylic acid	
Formula:	C_{16} H_{18} F N_5 O_2	
MW, mean [g/mol]:	331.35	335.32
Certificate of analysis:	AZ 13481	K-1654
Purity:	98.2 %	96.7 %
Expiry date:	July 2008	
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

report/standard name	**AE1170437 Triazine-indanone**	**AE1170437-ketone-triazine-$^{15}N_4$ (ISTD)**
		
IUPAC-name	(2R,3R)-3-({4-amino-6-[(1R)-1-fluoroethyl]-1,3,5-triazin-2-yl}amino)-2,5-dimethylindan-1-one	
Formula:	C_{16} H_{18} F N_5 O	
MW, mean [g/mol]:	315.35	319.32
Certificate of analysis:	AZ 12812	K-1639
Purity:	95 %	94.9 %
Expiry date:	July 2007	
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

report/standard name	**AE1170437 Hydroxyethyl**	**AE1170437-desfluorohydrxy-triazine-$^{15}N_4$, (ISTD)**
		
IUPAC-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	1-(4-amino-6-{[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]amino}-1,3,5-triazin-2-yl)ethanol
Formula:	$C_{16}H_{21}N_5O$	
MW, mean [g/mol]:	299.38	303.34
Certificate of analysis:	AZ 13136	K-1650
Purity:	99.1 %	97.6 %
Expiry date:	January 2008	
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

report/standard name	AE1170437 Olefin	**AE1170437-olefin-triazine-$^{15}N_4$, (ISTD)**
		
IUPAC-name	N-[(1R,2S)-2,6-dimethyl-2,3-dihydro-1H-inden-1-yl]-6-vinyl-1,3,5-triazine-2,4-diamine	
Formula:	$C_{16}H_{19}N_5$	
MW, mean [g/mol]:	281.36	285.33
Certificate of analysis:	AZ 13730	K-1651
Purity:	96.0 %	97.5 %
Expiry date:	October 2008	
Origin:	Bayer CropScience GmbH, PT– Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

report/standard name	**AE1170437 Diaminotriazine**	**AE1170437 1-Fluoroethyl triazinediamine-$^{15}N_5$, $^{13}C_2$ (ISTD)**
		
IUPAC-name	6-[(1R)-1-fluoroethyl]-1,3,5-triazine-2,4-diamine	
Formula:	$C_5 H_8 F N_5$	
MW, mean [g/mol]:	157.15	164.09
Certificate of analysis:	AZ 12734	K-1627
Purity:	93.5 %	97.4 %
Expiry date:	June 2007	
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany	Bayer CropScience GmbH, 17745 South Metcalf, Stilwell, KS 66085-9104, USA

3.2 Test System

The method was validated using two German soils from *Höfchen* and *Laacher Hof*. Two different soils were used in order to assess a possible influence of different soil characteristics. The soil samples were classified according to DIN and/or USDA specifications. Soil characteristics of the used soils are summarized below (for complete soil parameterization see Table 5):

Soil	Texture of Soil	Organic Matter [%]
Höfchen	silt loam (USDA)	1.58
Laacher Hof	sandy loam (USDA)	2.06

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 HPLC:

Number of the method: DH-002-S06-01

Title of the method: 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

Author of the method: Tianbo Xu, Robert J. Seymour, Bayer CropScience LP17745 S Metcalf Avenue, Stilwell, Kansas 66085

Limit of quantitation: 1.5 µg/kg

Table 1: Level and Number of Recoveries per Fortification Level

Soil	Control sample	Level 1.5 µg/kg	Level 15 µg/kg
Höfchen	2	5	5
Laacher Hof	2	5	5

Additionally, one solvent blank with internal standard added was analysed.

4.1.1 Outline of the Method

AE1170437 and its metabolites are extracted from soil and sediment with 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 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.

4.1.2 Instruments

Microwave Extractor: MLS-Ethos,
MWS Vertriebs GmbH
88299 Leutkirch, Germany

Balances: XP603S and XP205
Mettler Instruments GmbH
35387 Giessen, Germany

Ultrasonic Bath: Transsonic 890/H
Heinrich Faust
51145 Cologne, Germany

HPLC: HP 1100 Column Compartment G1316A, HP 1100 Binary Pump G1312A, HP 1100 Isocratic Pump G1310A, HP 1100 Degasser G1322A
Agilent
40880 Ratingen, Germany

Auto sampler: HTC PAL System
CTC Analytics AG
4222 Zwingen, Switzerland

Mass Spectrometer: API 4000 with turbo-ionspray source, PE Sciex Instruments 64331 Weiterstadt, Germany

Note: Instrument specific conditions of mass spectrometer were optimized by a skilled operator prior to analysis.

4.1.3 Reagents and Equipment

Column (HPLC):	Synergy Fusion-RP, 250 mm x 2 mm, Particle size 4 µm, pore size 80A, Cat. No. 00G-4424-B0, Phenomenex 63741 Aschaffenburg, Germany
Magnetic stirring bar:	plain (large, e.g. 40 x 8 mm [length x i.d.])
Acetonitrile:	for HPLC, super gradient grade Riedel de Haen, No. 34998 30926 Seelze, Germany
Methanol:	for residue analysis Promochem 46485 Wesel, Germany
Formic acid:	p.a. (100%) Merck, No.1.00264.0100 64271 Darmstadt, Germany
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 Chromatographic Conditions and Mass Spectrometric Parameters

Liquid chromatographic conditions were identical to those described in Appendix 1 of the original method report; MS/MS parameter settings were optimized for the instrument being used and therefore not identical with those reported in method DH-002-S06-01.

4.1.5 Calculation

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystems quantitation software Analyst (vers. 1.4) using linear regression. Further calculations were performed using the software EXCEL 2002 (Office 2002®).
Matrix effects for AE1170437 and its metabolites are eliminated by using an internal standard solution of the isotopically labeled reference items. Generally, the concentration of the internal standards should be in the range of the concentration of the analytes in the sample solutions.

The linear equation is expressed as:

$$y = \text{Intercept} + \text{Slope} \cdot x$$

y = Area, x = Concentration

If an internal standard is used:

$$y = \frac{\text{Area}_{\text{Standard}}}{\text{Area}_{\text{Internal Standard}}} = \text{Int. Ratio} \quad \text{and} \quad x = \frac{\text{Conc}_{\text{Standard}}}{\text{Conc}_{\text{IS}}} = \text{Conc}_{\text{ratio}}$$

Int. Ratio: *intensity ratio*

$Conc_{Standard}$: *concentration of standard solution [µg/L]*

$Conc_{IS}$: *concentration of internal standard solution [µg/L]*

$Conc_{ratio}$: *concentration ratio*

Since the concentrations of the isotopically labeled internal standards were the same in all sample and standard solutions that were injected into the HPLC instrument, their concentrations can be neglected for calculations. However, the unit (µg/L or ng/mL) of the internal standard concentration has always to be considered in the calculation while the value is set to 1 (see example calculation). In cases were the concentration is taken into account in the formula for $Conc_{ratio}$, it has to appear in the formula for $Conc_{Soil}$, too.
By means of the linear equation, the compounds concentration in soil can be calculated as follows:

$$\text{Dilution}_{\text{Factor}} = \frac{\text{Volume}_{\text{Extraction}}}{\text{Weight}}$$

$$\text{Conc}_{\text{Analyte}} = \frac{\text{Int.Ratio} - \text{Intercept}}{\text{Slope}}, \text{ Int. Ratio} = \frac{\text{Area}_{\text{Analyte}}}{\text{Area}_{\text{Internal Standard}}}$$

$$\text{Conc}_{\text{Soil}} = \text{Conc}_{\text{Analyte}} \times \text{Dilution}_{\text{Factor}} \times \text{Conc}_{\text{IS}}$$

$Volume_{Extraction}$: *volume of the extraction solvent [L]*

Weight: *weight of the soil sample [kg]*

Intercept: *intercept of the linear regression curve*

Slope: *slope of the linear regression curve*

Area $_{Analyte}$: *area of the analyte in the sample solution*

$Conc_{Soil}$: *concentration of the analyte in soil (sediment) [µg/kg]*

The recovery is calculated according to the following equation:

$$\text{Recovery} = \frac{\text{Conc}_{\text{Soil (Sediment)}} \times 100\%}{\text{Conc}_{\text{Soil (Sediment) Spiked}}}$$

$Conc_{Soil\ (Sediment)\ Spiked}$: *concentration of the reference item spiked [µg/kg]*

4.1.6 Deviations from the Method

No significant changes were made to the original method DH-002-S06-01.
Only the Q3-mass for the confirmatory method of AE1170437 Diaminotriazine was changed to 96 amu, because the proposed Q3-mass of 85 amu did not result in any selectivity. Since the validation of a confirmatory method was not subject of this ILV, this fact is only mentioned for completeness.

4.2 Linearity of the Detector

The linearity of the detector response for AE1170437 and its metabolites were tested by injections of standard solutions with the following concentrations:

Table 2: Standard Concentrations for the Determination of Detector Linearity

	Concentration [µg/L]				
AE1170437	0.05	0.1	0.5	1	5
AE1170437 Carboxylic Acid	0.05	0.1	0.5	1	5
AE1170437 Triazine-indanone	0.05	0.1	0.5	1	5
AE1170437 Hydroxyethyl	0.05	0.1	0.5	1	5
AE1170437 Olefin	0.05	0.1	0.5	1	5
AE1170437 Diaminotriazine	0.05	0.1	0.5	1	5