2. BACKGROUND

AE C656948 is a fungicide currently being developed by Bayer CropScience with potential uses in several crops.

The purpose of this study was to demonstrate that the "Analytical Method 01023 for the Determination of Residues of AE C656948 and its Metabolites AE C656948-benzamide (AE F148815), AE C656948-7-hydroxy (BCS-AA-10065) and AE C656948-PCA in Soil by HPLC-MS/MS"[1], can be performed with acceptable recoveries at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience AG, Development-Residues, Operator and Consumer Safety, at their laboratory in Monheim, Germany and reported by Thomas Freitag.

This study was performed in accordance with US EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue Analytical Method, August 1996 [2], and US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996 [3].

In addition to performing this independent laboratory validation (ILV) on soil, a sediment sample was included to determine the suitability of the method to analyze AE C656948 and its metabolites in sediment.

The method extraction was performed as written. The LC/MS/MS parameter settings were in general as described in method 1023 but optimized for the instrument being used.

The method was successfully validated for AE C656948 and its Metabolites AE C656948-benzamide (AE F148815), AE C656948-7-hydroxy (BCS-AA-10065) and AE C656948-PCA in both soil and sediment.

On completion of this study the method was reissued to include both soil and sediment and assigned a method number of GM-002-S07-01[4].

3. EXPERIMENTAL DETAILS

Study initiation date: May 21, 2007
Experimental Completion Date: August 2, 2007

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

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3.1 Test Substances

The test substances for this study were AE C656948 and its metabolites AE C656948-benzamide (AE F148815), AE C656948-7-hydroxy (BCS-AA-10065) and AE C656948-PCA. See Appendix 3 for complete nomenclature and chemical structures.

3.2 Analytical Reference Substances

The test substances also served as the analytical reference substances. See Appendix 3 for complete nomenclature, chemical structures and reference information for the reference substances. The test and reference substances were stored in a freezer until used to prepare fortification and calibration solutions. All stock solutions were stored in a freezer set at an average temperature of -18℃ when not in use. All fortifica tion and calibration solutions were stored in a refrigerator set at an average temperature of 7℃ when not in use.

3.3 Test System

The test systems were sub-samples of bulk untreated soil and sediment samples. The soil samples were obtained from Bayer CropScience Study Numbers MEGMP086 [5], Terrestrial Field Dissipation of AE C656948 in a Georgia Soil, 2006 (Sample ID MEGMP086-A10104-NUT1) and MEGMP087 [6], Terrestrial Field Dissipation of AE C656948 in a California Soil, 2006 (Sample ID MEGMP087-A10104-NUT1). The sediment samples were obtained from Bayer CropScience Study Number MEELP008 [7], Anaerobic Aquatic Metabolism of [^{14}C]-BYF 14182 (Sample Number 040507-D). Characterization data for each of these samples is presented in Appendix 4.

The soil and sediment samples were stored at room temperature prior to fortification.

3.4 Method Summary

Each analytical set included one reagent blank, two unfortified control samples, five samples fortified at the LOQ (1.0ng/g or ppb) and five samples fortified at 10x LOQ (10.0ng/g)

AE C656948 and its metabolites were extracted from soil and sediment using microwave extraction. An isotopic internal standard was added to the extract, which was evaporated to dryness, reconstituted in water:acetonitrile with acetic acid and analyzed by LC/MS/MS for AE C656948, AE C656948-benzamide, AE C656948-7-hydroxy and AE C656948-PCA.

3.5 Instrumentation

- Sciex API 4000 LC/MS/MS System (Applied Biosystems)
- Shimadzu LC-10AD$_{VP}$ HPLC Pumps (2) with a high pressure mixer and Shimadzu SCL-10A$_{VP}$ Pump Controller
- Perkin Elmer 200 Series Autosampler

3.6 LC/MS/MS Conditions

Note that the gradient parameters and MS settings listed below for the API 4000 MS/MS are different from the original method which used an API 3000 MS/MS, but found to be optimum for the LC/MS/MS instrument used in this study.

Two separate analyses were performed, AE C656948, AE C656948-benzamide (AE F148815), AE C656948-7-hydroxy (BCS-AA-10065) were quantified using one set of LC/MS/MS conditions and AE C656948-PCA using a second set of LC/MS/MS conditions.

AE C656948, AE C656948-benzamide (AE F148815), AE C656948-7-hydroxy (BCS-AA-10065) analysis

Column: Eclipse XDB-C8, 3.5 µm, length 150 mm, i.d. 2.1 mm
Agilent Technologies P.N. 930990-906

Injection volume: 5 µL
Mobile phase: A: Water/acetic acid (1000:5, v,v)
B: Acetonitrile/acetic acid (1000:5, v,v)
Flow rate (column): 0.200 mL/min

Retention times: AE C656948-benzamide: approx. 3.7 minutes
AE C656948-7-hydroxy approx. 5.2 minutes
AE C656948: approx. 5.7 minutes

HPLC Gradient Parameters

Time [min]	% A	% B
0.0	70	30
1.0	60	40
3.0	20	80
3.3	10	90
6.5	10	90
7.0	70	30
7.5	70	30
9.5	System Controller	Stop

MS/MS Conditions

CAD (Collision Gas)	7
CUR (Curtain Gas) [psi]	35
GS1 (Ion Source Gas 1) [psi]	20
GS2 (Ion Source Gas 2) [psi]	60
Source Temperature [°C]	600
Ihe (interface heater)	ON
Ion Transfer Voltage (IS) [V]	5500
Resolution of Q1 and Q3	Q1 Unit, Q3 Low
Scan type	MRM
Polarity	Positive
Ion Source	Turbo Spray

	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)
AE C656948 Quantitation	397	173	500	66	10	41	16
AE C656948 Confirmatory	397	208	500	66	10	33	12
AE C656948 ^{13}C Internal Standard	403	179	500	76	10	45	32
AE C656948-benzamide Quantitation	190	130	500	40	9	30	12
AE C656948-benzamide Confirmatory	190	170	500	40	9	15	14
AE C656948-benzamide ^{13}C Internal Standard	196	135	300	46	9	29	14
AE C656948-7-hydroxy Quantitation	413	173	300	71	10	37	55
AE C656948-7-hydroxy Confirmatory	413	145	300	71	10	75	28
AE C656948-7-hydroxy ^{13}C Internal Standard	419	179	500	81	10	41	18

The ^{13}C isotope transitions are used for both the quantitation and confirmatory analyses

Detector Parameters	
Channel Electron Multiplier (CEM)	2250 V
Deflector (DF)	-100 V

AE C656948-PCA analysis

Column: Eclipse XDB-C8, 3.5 µm, length 150 mm, i.d. 2.1 mm
Agilent Technologies P.N. 930990-906

Injection volume: 20 µL

Mobile phase: A: Water/acetic acid (1000:5, v,v)
B: Acetonitrile/acetic acid (1000:5, v,v)
Flow rate (column): 0.200 mL/min

Retention times: AE C656948-PCA : approx. 4.7 minutes

HPLC Gradient Parameters

Time [min]	% A	% B
0.0	90	10
0.7	90	10
3.0	10	90
5.0	10	90
5.1	90	10
7.5	System Controller	Stop

MS/MS Conditions

CAD (Collision Gas)	7
CUR (Curtain Gas) [psi]	35
GS1 (Ion Source Gas 1) [psi]	20
GS2 (Ion Source Gas 2) [psi]	60
Source Temperature [°C]	700
Ihe (interface heater)	ON
Ion Transfer Voltage (IS) [V]	4500
Resolution of Q1 and Q3	Q1 Unit, Q3 Low
Scan type	MRM
Polarity	Negative
Ion Source	Turbo Spray

	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)
AE C656948-PCA Quantitation	224	180	300	-25	-9	-14	-11
AE C656948-PCA Confirmatory	224	162	300	-25	-9	-14	-12
AE C656948-PCA ^{13}C Internal Standard	228	183	300	-25	-9	-14	-11

The ^{13}C isotope transitions are used for both the quantitation and confirmatory analyses

Detector Parameters	
Channel Electron Multiplier (CEM)	2250 V
Deflector (DF)	325 V

3.7 Calculations

An example calculation for AE C656948 from sample RAGMP101-set 3-LOQ1, which was analyzed during the method validation study, is shown below. This sample was fortified with 1ng/g of AE C656948, AE C656948-benzamide, AE C656948-7-hydroxy and AE C656948-PCA. The chromatogram used in this example is presented in Appendix 2 (Chromatogram 5).

The standards were fit to the linear equation: $Y = MX + B$

where: X is the concentration of the reference standard in ng/mL
M is the calibration line slope
B is the calibration line intercept
Y is the native peak area:isotopic peak area ratio

The example shown below is for the calculation of AE C656948 residues. AE C656948-benzamide, AE C656948-7-hydroxy and AE C656948-PCA residues are calculated in a similar fashion.

After regression coefficients were calculated, the residue in ng/g was determined. The ng/g of AE C656948 in the soil was calculated using the following equation,

$$\text{AE C656948 found (ng/g)} = \frac{(Y-B) \times D}{M}$$

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

W	V_1	V_2	V_3	Native Peak Area	IS Peak Area	**Y**	**M**	**B**
20g	40mL	1mL	1mL	30837.1	131697.2	0.2342	0.5226	9.75×10^{-7}

The slope and intercept were obtained from the calibration curve generated by Analyst, and is presented in Appendix 1 (Figure 1). The calibration points were weighted 1/x to provide better fit near the limit of detection

From the above equations:

$$\text{Dilution Factor (D)} = \frac{40}{20} \times \frac{1}{1} = 2$$

$$\text{AE C656948 found} = \frac{(0.2342 - (9.75 \times 10^{-7})) \times 2}{0.5226} = 0.896 \text{ng/g}$$

Therefore sample RAGMP101-set 3-LOQ1 contains 0.896ng/g AE C656948.

The % recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{R}{T} \times 100$$

Where: R = ng/g of target analyte found in fortified sample
T = theoretical ng/g in fortified sample

Therefore, for sample RAGMP101-set 3-LOQ1,

R = 0.896ng/g
T = 1.0 ng/g

$$\text{\% Recovery} = \frac{(0.896)}{1.0} \times 100 = 90\%$$

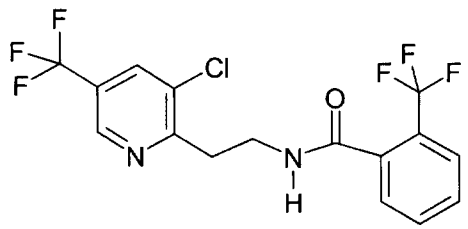
Remark: Example calculations shown above were performed using the LC/MS/MS software *Analyst (version 1.4.1)*. The example calculation was performed using the area values reported by the instrument. The instrument software carries additional figures not shown in the intermediate results. Therefore, instrument software calculated values will differ slightly from the results derived using a calculator.

Appendix 3. Identity and Purity of the Test and Reference Materials Used

AE C656948

Active Substance:	AE C656948
Chemical Name:	N-{2-(3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl) benzamide
Empirical Formula:	$C_{16}H_{11}CLF_6N_2O$
Molecular Mass:	397 g/mol
Standard No:	K-1377
Expiry Date:	05/15/12
Purity:	99.8 %
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

Structural Formula:



AE C656948-benzamide (AE F148815)

Active Substance:	AE C 656948-benzamide
Chemical Name:	2-trifluoromethylbenzamide
Empirical Formula:	$C_8H_6F_3NO$
Molecular Mass:	189 g/mol
Standard No.:	K-1619
Expiry Date:	01/31/08
Purity:	99.0%
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

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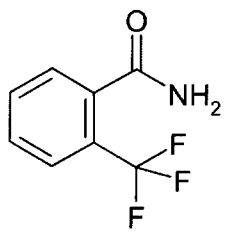
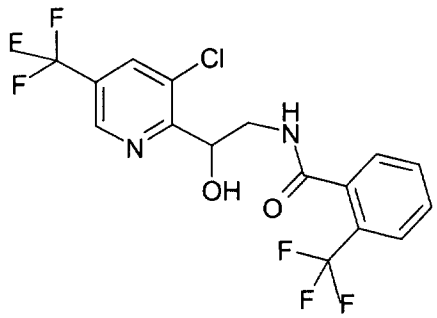


AE C656948-7-hydroxy (BCS-AA10065)

Active Substance:	AE C656948-7-hydroxy
Chemical Name:	N-{2-(3-chloro-5-(trifluoromethyl)pyridin-2-yl]-2-hydroxyethyl}-2-(trifluoromethyl)benzamide
Empirical Formula:	$C_{16}H_{11}ClF_6N_2O_2$
Molecular Mass:	413 g/mol
Standard No.:	K-1677
Expiry Date:	05/18/08
Purity:	99 %
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

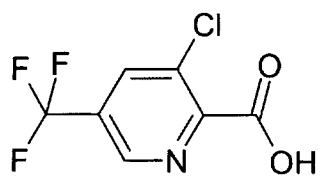
Structural Formula:



AE C656948-PCA (AE C657188)

Active Substance:	AE C656948-PCA
Chemical Name:	3-chloro-5-trifluoromethylpyridine-2-caboxylic acid
Empirical Formula:	$C_7H_3ClF_3NO_2$
Molecular Mass:	226 g/mol
Standard No.:	K-1185
Expiry Date:	04/14/08
Purity:	97.2 %
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

Structural Formula:

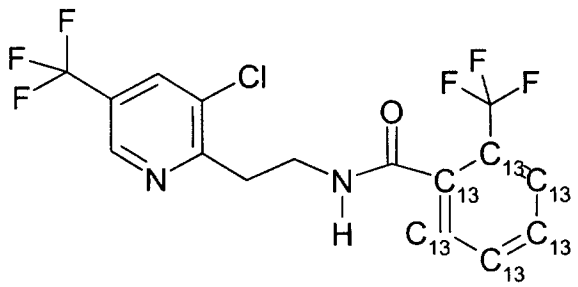


Internal Standards

AE C656948-phenyl-$^{13}C_6$

Active Substance:	[phenyl-$^{13}C_6$] AE C656948
Chemical Name:	N-{2-(3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl) benzamide
Empirical Formula:	$^{13}C_6C_{10}H_{11}ClF_6N_2O$
Molecular Mass:	403 g/mol
Standard No:	K-1564
Expiry Date:	10/12/15
Purity:	100
Origin:	Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

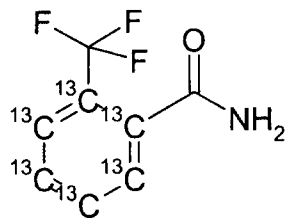
Structural Formula:



[phenyl-$^{13}C_6$] AE C656948-benzamide (AE F148815-phenyl-$^{13}C_6$)

Active Substance:	[phenyl-$^{13}C_6$] AE C656948-benzamide
Chemical Name:	2-(trifluoromethyl)benzamide
Empirical Formula:	$^{13}C_6C_2H_6F_3NO$
Molecular Mass:	195 g/mol
Standard No:	K-1672
Expiry Date:	03/14/17
Purity:	96.6 %
Origin:	Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

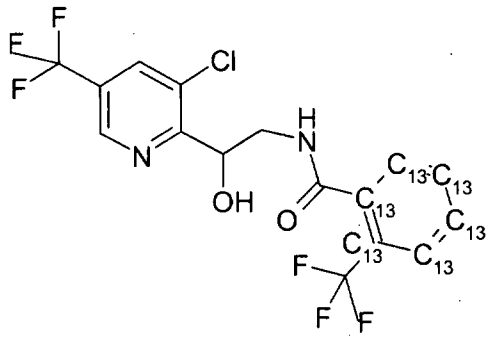
Structural Formula:



[phenyl-$^{13}C_6$] AE C656948-7-hydroxy (BCS-AA10065--phenyl-$^{13}C_6$)

Active Substance:	[phenyl-$^{13}C_6$] AE C656948-7-hydroxy
Chemical Name:	N-{2-(3-chloro-5-(trifluoromethyl)pyridin-2-yl]-2-hydroxyethyl}-2-(trifluoromethyl)benzamide
Empirical Formula:	$^{13}C_6C_{10}H_{11}ClF_6N_2O_2$
Molecular Mass:	418.6g/mol
Standard No:	K-1671
Expiry Date:	02/26/17
Purity:	100%
Origin:	Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

Structural Formula:



[2,6-^{13}C, ^{15}N;carboxylic acid-^{13}C] AE C656948-PCA (AE C657188-2,6-^{13}C)

Active Substance:	[2,6-^{13}C, ^{15}N;carboxylic acid-^{13}C] AE C656948-PCA
Chemical Name:	3-chloro-5-trifluoromethylpyridine-2-caboxylic acid
Empirical Formula:	$^{13}C_3C_4H_3ClF_3{}^{15}NO_2$
Molecular Mass:	229.5 g/mol
Standard No:	K-1673
Expiry Date:	03/14/17
Purity:	97.0%
Origin:	Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

Structural Formula: