

2 Introduction and Objective

The objective of the study was to validate a new residue analytical method for the analysis of the active ingredient AE C656948 and its metabolites AE C656948-benzamide, AE C656948-7-hydroxy and AE C656948-PCA in soil based on HPLC-MS/MS. The method was developed in accordance to the Guidance Document on Residue Analytical Methods [1], BBA Guideline for Residue Analytical Methods [2] and the Commission Directive 96/46/EC [3].

3 Compounds

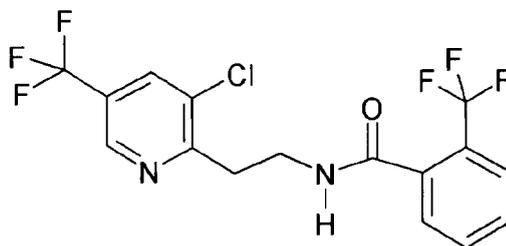
3.1 Reference Item

Generally, only sufficiently characterised and certified substances were used as reference items.

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: 396.72 g/mol
Analysis Certificate: AZ 12477
Expiry Date: April 2007
Purity: 99.8 %
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt,
D-65926 Frankfurt am Main, Germany

Structural Formula:

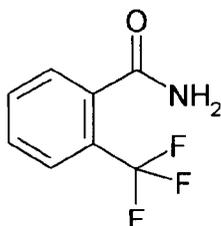


3.1 Reference Item (contd)

AE C656948-benzamide

Active Substance: AE C 656948-benzamide
Chemical Name: 2-trifluoromethylbenzamide
Empirical Formula: $C_8H_6F_3NO$
Molecular Mass: 189.15 g/mol
Analysis Certificate: AZ 13174
Expiry Date: January 2008
Purity: 99.0 %
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt,
D-65926 Frankfurt am Main, Germany

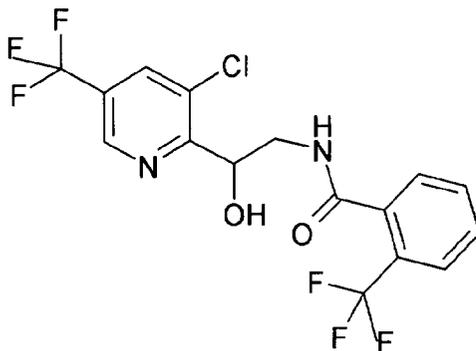
Structural Formula:



AE C656948-7-hydroxy

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: 412.72 g/mol
Analysis Certificate: AZ 13407
Expiry Date: May 2008
Purity: 99 %
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt,
D-65926 Frankfurt am Main, Germany

Structural Formula:

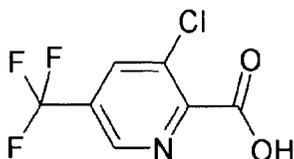


3.1 Reference Item (contd)

AE C656948-PCA

Active Substance: AE C656948-PCA
Chemical Name: 3-chloro-5-(trifluoromethyl)pyridine-2-carboxylic acid
Empirical Formula: $C_7H_3ClF_3NO_2$
Molecular Mass: 225.55 g/mol
Analysis Certificate: AZ 13316
Expiry Date: April 2008
Purity: 97.2 %
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt,
D-65926 Frankfurt am Main, Germany

Structural Formula:

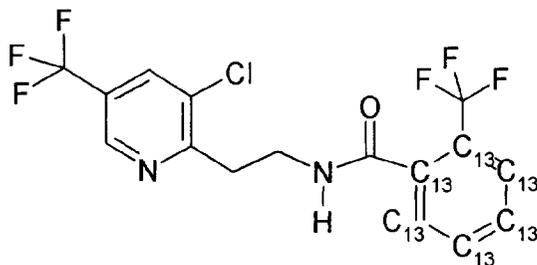


3.2 Internal Standard

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

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: 402.66 g/mol
Batch-Number: BECH 1227-1-1
Purity: >99 %
Origin: Bayer CropScience GmbH, Research-Product Technology Isotope Chemistry,
D-42046 Wuppertal

Structural Formula:

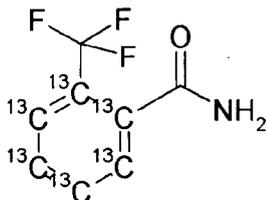


3.2 Internal Standard (contd)

[phenyl-¹³C₆] AE C656948-benzamide

Active Substance: [phenyl-¹³C₆] AE C656948-benzamide
Chemical Name: 2-(trifluoromethyl)benzamide
Empirical Formula: ¹³C₆C₂H₆F₃N O
Molecular Mass: 195.07 g/mol
Batch-Number: KML 3611-1-14
Purity: >99 %
Origin: Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

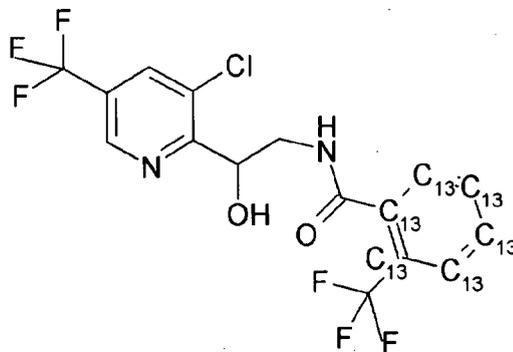
Structural Formula:



[phenyl-¹³C₆] AE C656948-7-hydroxy

Active Substance: [phenyl-¹³C₆] AE C656948-7-hydroxy
Chemical Name: N-{2-(3-chloro-5-(trifluoromethyl)pyridin-2-yl)-2-hydroxyethyl}-2-(trifluoromethyl)benzamide
Empirical Formula: ¹³C₆C₁₀H₁₁ClF₆N₂O₂
Molecular Mass: 418.66 g/mol[^]
Batch-Number: BECH 1309-2-5
Purity: >98.9 %
Origin: Bayer CropScience GmbH, Researche-Product Technology Isotope Chemistry, D-42046 Wuppertal

Structural Formula:

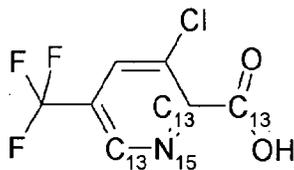


3.2 Internal Standard (contd)

[2,6-¹³C, ¹⁵N;carboxylic acid-¹³C] AE C656948-PCA

Active Substance: [2,6-¹³C, ¹⁵N;carboxylic acid-¹³C] AE C656948-PCA
Chemical Name: 3-chloro-5-trifluoromethylpyridine-2-carboxylic acid
Empirical Formula: ¹³C₃H₃ClF₃¹⁵NO₂
Molecular Mass: 229.52 g/mol[^]
Batch-Number: BECH 1340-1-3
Purity: >98 %
Origin: Bayer CropScience GmbH, Research-Product Technology Isotope Chemistry,
D-42046 Wuppertal

Structural Formula:



4 Experimental Section

4.1 Test System

The method was validated using the two German soils 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 summarised in Table 9. Complete soil characterisation is reported in the Appendix 6.

Table 9: Soil Types

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

4.2 Safety

While processing this method, the German guidelines for laboratories of the professional association (e.g. leaflet M006 [4]) or comparable guidelines in other countries are to be considered.

The following chemicals were used, which are classified by the hazardous material regulations. The classification is based on the German guidelines [5] and has to be adapted to the respective national guidelines in case the method is used outside Germany.

AE C656948	Very toxic* T+
[phenyl- ¹³ C ₆] AE C656948	Very toxic* T+
AE C 656948-benzamide	Very toxic* T+
[phenyl- ¹³ C ₆] AE C 656948-benzamide	Very toxic* T+
AE C656948-7-hydroxy	Very toxic* T+
[phenyl- ¹³ C ₆] AE C656948-7-hydroxy	Very toxic* T+
AE C 656948-PCA	Very toxic* T+
[2,6- ¹³ C, ¹⁵ N;carboxylic acid- ¹³ C] AE C 656948-PCA	Very toxic* T+
Acetonitrile	Harmful Xn, highly flammable F
Acetic acid	Corrosive C

* A classification is not yet available. Due to this fact the compound has to be treated as very toxic substance.

The pertinent safety instructions must be observed when working with all compounds mentioned in this method (e.g. R- and S-phrases). It has to be made sure that the working place is properly ventilated when working with dry ice. Sample vessel and deep-freezing cabinet must guarantee pressure equalisation.

4.3 Materials

4.3.1 Apparatus and Reagents

For apparatus and reagents please see Appendix 1.

4.3.2 Stock Solutions

In Table 10, Table 11, and Table 12 are general working procedures for the preparation of standard solutions described. Therefore, given names, weights and volumes do not correspond exactly to the used names, weights, volumes and concentrations documented in the raw data.

The stock solution was prepared by weighing a defined amount of reference item into a volumetric flask and making up to volume with acetonitrile (ACN).

Table 10: Preparation Scheme of Reference Item Stock Solution

Reference Item No.	Reference Item	Mass	Volume	Solvent	Final Concentration	
		[mg]	[mL]		Required [mg/L]	Actual [mg/L]
1	AE C656948 [STM948]	11.06	20	ACN	500	552
2	AE C656948 - benzamide [STM815]	12.58	20	ACN	500	623
3	AE C656948 -7- hydroxy [STM065]	10.29	20	ACN	500	509
4	AE C656948 - PCA [STM188]	12.34	20	ACN	500	600
5	[phenyl- ¹³ C ₆] AE C656948 [STM48C]	5.00	50	ACN	100	99
6	[phenyl- ¹³ C ₆] AE C656948-benzamide [STM15C]	5.09	50	ACN	100	101
7	[phenyl- ¹³ C ₆] AE C656948-7-hydroxy [STM65C]	4.42	10	ACN	500	438
8	[2,6- ¹³ C, ¹⁵ N;carboxylic acid- ¹³ C] AE C656948 -PCA [STM88C]	5.00	50	ACN	100	98

Before further use, the standard stock solution have to be ultrasonicated for about one minute to achieve complete dissolution of the test item.

4.3.3 Standard Solutions

Standard solutions (secondary standards) were prepared from the stock solution by dilution with acetonitrile/water/acetic acid.

Table 11: Preparation Scheme for Reference Standards

No.	Reference-Substances	Concentration [ng/mL]	No. of Solution	Aliquot [mL]	Dilution to [mL]	Solvent
1 LSG948	AE C656948	10000	STM948	1.812	100	*
2 LSG815	AE C656948-benzamide	10001	STM815	1.606	100	*
3 LSG065	AE C656948-7-hydroxy	10004	STM065	1.964	100	*
4 LSG188	AE C656948-PCA	10003	STM188	1.668	100	*
5 LSG948	AE C656948	400	1 LSG948	2	50	*
6 LSG815	AE C656948-benzamide	400	2 LSG815	2	50	*
7 LSG065	AE C656948-7-hydroxy	400	3 LSG065	2	50	*
8 LSG188	AE C656948-PCA	400	4 LSG188	2	50	*
9 LSG948	AE C656948	4	5 LSG948	1	100	*
10 LSG815	AE C656948-benzamide	4	6 LSG815	1	100	*
11 LSG065	AE C656948-7-hydroxy	4	7 LSG065	1	100	*
12 LSG188	AE C656948-PCA	4	8 LSG188	1	100	*
13 LSG948	AE C656948	4	5 LSG948	1	100	**
14 LSG815	AE C656948-benzamide	4	6 LSG815	1	100	**
15 LSG065	AE C656948-7-hydroxy	4	7 LSG065	1	100	**
16 LSG188	AE C656948-PCA	4	8 LSG188	1	100	**

*: Acetonitrile/water/acetic acid (500/500/1, v/v/v).

** : Acetonitrile/water/acetic acid (100/900/5, v/v/v).

4.3.4 Calibration Standards

Standard mixture solutions were prepared by dilution with acetonitrile/water/acetic acid mixture (100/900/5, v/v/v). These standards were used to run the linearity investigations and for quantitation of residues.

Table 12: Preparation Scheme for Calibration Standards

No.	Reference-Substances	Concentration [ng/mL]	No. of Solution	Aliquot [mL]	Dilution to [mL]
1 MIX948	AE C656948	10000	1	1.812	100
	AE C656948-benzamide	10001	3	1.606	
	AE C656948-7-hydroxy	10004	5	1.964	
	AE C656948-PCA	10003	7	1.668	
2 MIX948*	AE C656948	1000	1 MIX948	10	100
	AE C656948-benzamide	1000			
	AE C656948-7-hydroxy	1000			
	AE C656948-PCA	1000			
3 MIX948*	AE C656948	100	2 MIX948	10	100
	AE C656948-benzamide	100			
	AE C656948-7-hydroxy	100			
	AE C656948-PCA	100			
4 MIX948	AE C656948	10	2 MIX948	1	100
	AE C656948-benzamide	10			
	AE C656948-7-hydroxy	10			
	AE C656948-PCA	10			
5 MIX948	13C6 AE C656948	3960	2	2	50
	13C6 AE C656948-benzamide	4031	4	2	
	13C6 AE C656948-7-hydroxy	4376	6	0.5	
	13C3 AE C656948-PCA	3920	8	2	
6 MIX948	13C6 AE C656948	238	5 MIX948	6	100
	13C6 AE C656948-benzamide	242			
	13C6 AE C656948-7-hydroxy	263			
	13C3 AE C656948-PCA	235			
7 MIX948	AE C656948	50.00	1 MIX948	0.5	100
	AE C656948-benzamide	50.00			
	AE C656948-7-hydroxy	50.02			
	AE C656948-PCA	50.02			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			
8 MIX948	AE C656948	40.00	1 MIX948	0.4	100
	AE C656948-benzamide	40.00			
	AE C656948-7-hydroxy	40.01			
	AE C656948-PCA	40.01			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			

*: This standard solution was used for fortification of the recoveries.

4.3.4 Calibration Standard (contd)

Table 12: Preparation Scheme for Calibration Standards (contd)

No.	Reference-Substances	Concentration [ng/mL]	No. of Solution	Aliquot [mL]	Dilution to [mL]
9 MIX948	AE C656948	20.00	1 MIX948	0.2	100
	AE C656948-benzamide	20.00			
	AE C656948-7-hydroxy	20.00			
	AE C656948-PCA	20.00			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			
10 MIX948	AE C656948	5.00	3 MIX948	5	100
	AE C656948-benzamide	5.00			
	AE C656948-7-hydroxy	5.00			
	AE C656948-PCA	5.00			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			
11 MIX948	AE C656948	0.50	3 MIX948	0.5	100
	AE C656948-benzamide	0.50			
	AE C656948-7-hydroxy	0.50			
	AE C656948-PCA	0.50			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			
12 MIX948	AE C656948	0.30	3 MIX948	0.3	100
	AE C656948-benzamide	0.30			
	AE C656948-7-hydroxy	0.30			
	AE C656948-PCA	0.30			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			

*: This standard solution was used for fortification of the recoveries.

4.3.4 Calibration Standard (contd)

Table 12: Preparation Scheme for Calibration Standards (contd)

No.	Reference-Substances	Concentration [ng/mL]	No. of Solution	Aliquot [mL]	Dilution to [mL]
13 MIX948**	AE C656948	0.15	3 MIX948	0.15	100
	AE C656948-benzamide	0.15			
	AE C656948-7-hydroxy	0.15			
	AE C656948-PCA	0.15			
	13C6 AE C656948	2.38	6 MIX948	1	
	13C6 AE C656948-benzamide	2.42			
	13C6 AE C656948-7-hydroxy	2.63			
	13C3 AE C656948-PCA	2.35			

** : This standard solution was used for the LOD estimation.

4.4 Sample Preparation

Extraction Procedure

1. Weigh 20 g of the soil sample into a 100-mL beaker containing a magnetic bar.
2. Add 40 mL of a mixture of acetonitrile/water (4:1, v/v).
3. Place always ten beakers with soil-solvent mixture into the microwave extractor.
4. Switch on the magnetic stirrer.
5. Extract for three minutes at 250 W. Always 10 beakers.
6. Add 400 µL of the internal standard solution (6 MIX948) and stir for approx. 1 min.
7. Transfer about 1.5 mL of the extract into a centrifuge tube. Centrifuge for 5 minutes at >12000 rpm (g) to remove fine particles of soil.
8. Transfer 1.0 ml of the centrifugal into culture tubes. Evaporate at 30°C to dryness using the Turbo Vap.
9. Add 1.0 ml of a mixture of water/acetonitrile/acetic acid (900/100/5 v/v/v).
10. Ultrasonic to complete dissolution
11. Transfer about 1 mL of the sample solution into an HPLC vial.
12. Inject an aliquot into the HPLC-MS/MS system.

A flow diagram of the analytical procedure is given in Appendix 2.

4.5 Instrumental Analysis

4.5.1 Principle of Measurement

An aliquot of the sample solution was injected into the high performance liquid chromatograph and subjected to reversed phase chromatography coupled with tandem mass spectrometry (MS/MS) with electrospray ionisation. The MS/MS instrument was operated in the Multiple Reaction Monitoring mode (MRM). The pseudomolecular ions of the analytes ($[M+H]^+$, $[M-H]^-$ or any adducts) were selected by the first quadrupole. These precursor ions were impulsed with nitrogen in the collision cell (second quadrupole) and the resulting fragment ions (product ions) were separated according to their m/z ratio in the third quadrupole. Two of these product ions per analyte were selected: one product ion (MRM-transition) serving for quantitation and the second for confirmation.

4.5.2 Variations in Instrument Conditions

Variations in equipment or sample characteristics and/or deterioration of system performance may require slight modifications in the chromatographic or detector conditions listed in order to obtain adequate chromatographic peak shapes or sensitivity. Instrument parameters and mobile phase may be adjusted to improve separation from unexpected interfering peaks.

Therefore, the given LC/MS/MS parameters listed may require adaptation.

4.5.3 Chromatography

Instrument: Agilent 3000 or equivalent
 Injector: HTC PAL, CTC Analytics or equivalent
 Column: Zorbax Eclipse XDB-C8, length 150 mm, i.d. 2.1 mm
 Agilent Technologies P.N. 930990-906, or equivalent
 Injection Volume: 25 µL or as needed for the sensitivity
 Oven temperature: 40 °C

Mobile Phase: Bin Pump A: water/acetic acid (1000:5, v,v,v)
 Bin Pump B: Acetonitrile/acetic acid (1000:5, v,v,v)
 Iso Pump C: water/acetonitrile/acetic acid (500:500:1, v,v,v)

Time Table:

Time [min]	A [%, v/v]	B [%, v/v]	Flow (Column) [mL/min]	Into MS	Into Waste
0.0	90	10	0.2	Iso pump	Bin pump
1.0	90	10	0.2		
1.1	10	90	0.2		
3.5				Bin pump	Iso pump
4.5	10	90	0.2		
4.6	90	10	0.2		
6.0				Iso pump	Bin pump
9.0	90	10	0.2		
9.0	Stop time				

Flow (into MS): 0.2 mL/min
 Retention time: AE C656948 approx. 5.2 min
 Retention time: [phenyl-¹³C₆] AE C656948 approx. 5.2 min
 Retention time: AE C656948-benzamide approx. 4.3 min
 Retention time: [phenyl-¹³C₆] AE C656948- benzamide approx. 4.3 min
 Retention time: AE C656948-7-hydroxy approx. 4.8 min
 Retention time: [phenyl-¹³C₆] AE C656948- 7-hydroxy approx. 4.8 min

4.5.3 Chromatography (contd)

Instrument: Agilent 3000 or equivalent
Injector: HTC PAL, CTC Analytics or equivalent
Column: Zorbax Eclipse XDB-C8; length 150 mm, i.d. 2.1 mm
Agilent Technologies P.N. 930990-906, or equivalent
Injection Volume: 100 µL or as needed for the sensitivity
Oven temperature: 40 °C

Mobile Phase: Bin Pump A: water/acetic acid (1000:5, v,v,v)
Bin Pump B: Acetonitrile/acetic acid (1000:5, v,v,v)
Iso Pump C: water/acetonitrile/acetic acid (500:500:1, v,v,v)

Time Table:

Time [min]	A [% v/v]	B [% v/v]	Flow (Column) [mL/min]	Into MS	Into Waste
0.0	90	10	0.2	Iso pump	Bin pump
1.0	90	10	0.2		
1.1	40	60	0.2		
4.0				Bin pump	Iso pump
7.5	40	60	0.2		
7.6				Iso pump	Bin pump
7.6	90	10	0.2		
15.0	90	10	0.2		
15.0	Stop time				

Flow (into MS): 0.2 mL/min
Retention time: AE C656948-PCA approx. 5.3 min
Retention time: [2,6-¹³C, ¹⁵N;carboxylic acid-¹³C] AE C656948-PCA approx. 5.3 min

For the LC/MS/MS parameters see Appendix 3

4.5.4 Detection

The detection by MS/MS was performed on a triple-quadrupole tandem mass spectrometer, equipped with a Turbo IonSpray (ESI) interface operated in positive ion and PCA negative mode and multiple reaction monitoring (MRM). Unit mass resolution was established and maintained in the mass resolving quadrupoles by maintaining a full width at half-maximum (FWHM) of about 0.7 amu. Optimal collisionally-activated dissociation (CAD) conditions for fragmentation of the pseudomolecular ions of the analytes and the corresponding stable isotopically labelled internal standards were applied with nitrogen as the collision gas.

Detector: Triple Quadrupole Tandem Mass Spectrometer, API3000 with turboionspray interface, mass selective detector (MS/MS), or any equivalent HPLC-MS/MS System

Interface: Turbo-IonSpray (ESI)
Gas Temperature: 350°C or as needed for the sensitivity

Scan Type: MRM (Multiple Reaction Monitoring)

Table 13: MS/MS Parameters for the Determination of AE C656948, AE C656948-benzamide, AE C656948-7-hydroxyde and AE C656948-PCA

	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)	Polarity
AE C656948 Quantitation	397	173	100	43	pos
AE C656948 Confirmatory	397	208	100	31	pos
AE C656948 isotop *	403	179	100	43	pos
AE C656948-benzamide Quantitation	190	130	100	29	pos
AE C656948-benzamide Confirmatory	190	170	100	17	pos
AE C656948-benzamide isotop *	196	136	100	29	pos
AE C656948-7-hydroxy Quantitation	413	173	100	79	pos
AE C656948-7-hydroxy Confirmatory	413	145	100	35	pos
AE C656948-7-hydroxy isotop *	419	179	100	79	pos
AE C656948-PCA Quantitation	224	180	300	-16	neg
AE C656948-PCA Confirmatory	224	162	300	-14	neg
AE C656948-PCA isotop *	228	183	300	-16	neg

* The isotop Transitions are used for quantification and confirmatory.

Note: Different MS/MS-instruments may result in different MRM transitions or signal intensity.

4.6 Calculation

The example calculation displayed below was used by the laboratory developing this method. Alternate calculation procedures appropriate to the reporting requirements may substitute the equations used below.

4.6.1 Calculation of Individual Residues and Recovery Rates

For calculation of the concentrations, calibration curves were used. These curves were calculated automatically after each sequence run with the Applied Biosystem quantitation software Analyst (Version 1.4) using linear regression. Further calculations were performed using the software EXCEL 2002 (Office 2002[®]).

The linear equation is expressed as:

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

y = Area, x = Concentration

If an internal standard is used:

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

<i>Int. Ratio:</i>	<i>intensity ratio</i>
<i>Conc_{Standard}:</i>	<i>concentration of standard solution [µg/L]</i>
<i>Conc_{IS}:</i>	<i>concentration of internal standard solution [µg/L]</i>
<i>Conc_{ratio}:</i>	<i>concentration ratio</i>

Since the concentration of the isotopically labelled internal standard was the same in all solutions that were injected into the HPLC instrument, its concentrations was neglected in the calculations. But if it would have been taken into account in the formula for $\text{Conc}_{\text{Ratio}}$, it would have to appear in the formula for $\text{Conc}_{\text{Soil Wet}}$, too.

However, the concentration of the isotopically labelled internal standard should be in the range of the concentration of the analyte in the sample solutions.

By means of the linear equation, the content of AE C656948 and BYF14182-3-hydroxybutyl in dry soil can be calculated as follows:

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

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

4.6.1 Calculation of Individual Residues and Recovery Rates (contd)

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

$$\text{Conc}_{\text{Soil Dry}} = \text{Conc}_{\text{Soil Wet}} \times \frac{100\%}{100\% - \text{Moisture}}$$

<i>Volume_{Extraction}:</i>	<i>volume of the extraction solvent [L]</i>
<i>Weight:</i>	<i>weight of the soil (sediment) sample [kg]</i>
<i>Intercept:</i>	<i>intercept of the linear regression curve</i>
<i>Slope:</i>	<i>slope of the linear regression curve</i>
<i>Area_{Analyte}:</i>	<i>area of the analyte in the sample solution</i>
<i>Moisture:</i>	<i>water content of the sample [kg/100kg]</i>
<i>Conc_{Soil Wet}:</i>	<i>concentration of the analyte in wet soil [$\mu\text{g}/\text{kg}$]</i>
<i>Conc_{Soil Dry}:</i>	<i>concentration of the analyte in dry soil [$\mu\text{g}/\text{kg}$]</i>

The recovery is calculated according to the following equation:

$$\text{Recovery} = \frac{\text{Conc}_{\text{Soil Wet}} \times 100\%}{\text{Conc}_{\text{Soil Spiked}}}$$

Conc_{Soil Spiked}: concentration of the reference substance spiked [$\mu\text{g}/\text{kg}$]

Example calculation for sample "Recovery 1 $\mu\text{g}/\text{kg}$ Höfchen 1 (m/z 179)":

$$\text{Dilution}_{\text{Factor}} = \frac{0.04\text{L}}{0.02\text{kg}} = 2 \frac{\text{L}}{\text{kg}}$$

$$\text{Conc}_{\text{Analyte}} = \frac{0.2346550 - (-0.00954769)}{1.1654} = 0.20954$$

$$\text{Conc}_{\text{Soil Wet}} = 0.20954 \times 2 \frac{\text{L}}{\text{kg}} \times 2.5 \frac{\mu\text{g}}{\text{L}} = 1.05 \frac{\mu\text{g}}{\text{kg}}$$

$$\text{Recovery} = \frac{1.05 \frac{\mu\text{g}}{\text{kg}} \times 100\%}{1.0 \frac{\mu\text{g}}{\text{kg}}} = 105\%$$

Remark: Calculations were performed using the computer software *Analyst (vers. 1.4)* and *EXCEL 2002*. The results given are rounded values. Thus, rounding deviations may occur if recalculations are made using the rounded figures.

4.6.2 Calculation of the Repeatability (Precision)

The repeatability or precision of the method is defined as the dispersion of the validation results and is expressed as the relative standard deviation (RSD).

At each fortification level, the relative standard deviation was calculated as follows:

$$\text{Relative Standard Deviation [\%]} = \frac{\text{Standard Deviation}}{\text{Mean of Recoveries}} \times 100$$

Appendix 1: Apparatus and Reagents

Apparatus

- Microwave Extractor, MLS-Ethos, MWS Vertriebs GmbH, 88299 Leutkirch, Germany or equivalent
- Balances, XP 205 and XP 603s Mettler Instruments GmbH, 35387 Giessen, Germany or equivalent
- Ultrasonic Bath, Transsonic 890/H, Heinrich Faust, 51145 Cologne, Germany or equivalent
- 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 or equivalent
- Autosampler, HTC PAL System, CTC Analytics AG, 4222 Zwingen, Switzerland or equivalent
- Mass Spectrometer, API 3000 with turbo-ionspray interface, Applied Biosystems, 64331 Weiterstadt, Germany or equivalent

Reagents

- Column, Zorbax Eclipse XDB-C8 3,5 μ , length 150 mm, i.d. 2.1 mm, Agilent Technologies P.N. 930990-906, Agilent 40880 Ratingen, Germany or equivalent
- Magnetic stirring bar, plain (large, e.g. 35 x 8 mm [length x i.d.]) or "dumb-bell" type (e.g. 35 x 8 mm [length x i.d.], diameter of end disk is 20 mm, from COWIE Technology, parts no. 1.1335) or equivalent
- Acetonitrile, for HPLC, super gradient grade, Riedel de Haen, No. 34998, D-30926 Seelze, Germany or equivalent
- Acetic acid p.a. Merck, D-64271 Darmstadt, Germany or equivalent
- Water, purified in a Milli-Q unit, Milli-Pore GmbH, D-65731 Eschborn, Germany or equivalent
- Volumetric flasks, pipettes and other equipment commonly used in the laboratory.

**Appendix 2:
Analytical Procedure of Method 01035**

Extraction Procedure

