

1 Summary

An independent laboratory validation (ILV) of the Residue Analytical Method Number AE-005-W05-02 "Method of Analysis for the Determination of Residues of AE 0172747 and its Metabolites AE 0456148 and AE 1392936 in Water Using LC/MS/MS" was conducted using surface water (taken from the River Rhine at Leverkusen-Hitdorf, Germany) and drinking water from the municipal water supply of Monheim, Germany.

The independent laboratory validation (ILV) was conducted using two untreated control waters from Germany (River Rhine Water and Tap Water Monheim) chosen as representative matrices. The analyses included a solvent blank with internal standards added, and a set of duplicate untreated controls, five control water samples fortified at the limit of quantitation (LOQ) and five control water samples fortified at 5× LOQ for both water qualities. The LOQ for all analytes was 0.05 µg/L.

The method was successfully validated for AE 0172747 and its metabolites AE 0456148 and AE 1392936.

2 Objective

The objective of this study was to demonstrate that method AE-005-W05-02 ("Method of Analysis for the Determination of Residues of AE 0172747 and its Metabolites AE 0456148 and AE 1392936 in Water Using LC/MS/MS") can be performed with acceptable recoveries for determination of the compounds AE 0172747, AE 0456148 and AE 1392936 at an independent laboratory having no prior experience with the method. The method was developed by Bayer CropScience LP, Stilwell, USA, and reported as Method AE-005-W05-02, by Robert J. Seymour & Diane Beck, in report dated May 24, 2005. River Rhine water (taken at Leverkusen-Hitdorf, Germany) and tap water Monheim, Germany were chosen as representative matrices for validation within the present study.

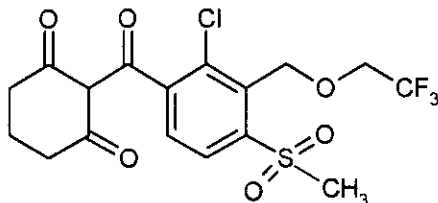
This study was performed in accordance with US EPA Ecological Effects Test Guidelines, OPPTS 850.7100 Data Reporting for Environmental Chemistry Methods, EPA 712-C-96-348, April 1996.

3 Materials

3.1 Test and Reference Items

AE 0172747:

Structural formula:



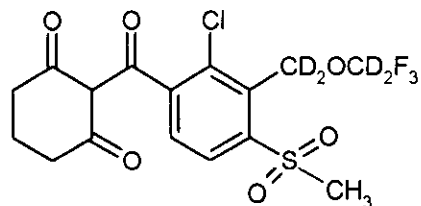
Chemical code:	AE 0172747
Chemical name (CAS):	2-{2-chloro-4-mesyloxy-3-[(2,2,2-trifluoroethoxy)methyl]benzoyl}cyclohexane-1,3-dione
Empirical formula:	C ₁₇ H ₁₆ Cl F ₃ O ₆ S
Molecular weight:	440.82 g/mol

Reference standard:

Certificate of analysis:	AZ 10961
Purity:	98.9 %
Expiry date:	August 2006
Origin:	Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

AE 0172747-trifluoroethoxymethyl-d4 (used as internal standard for the active ingredient AE 0172747):

Structural formula:

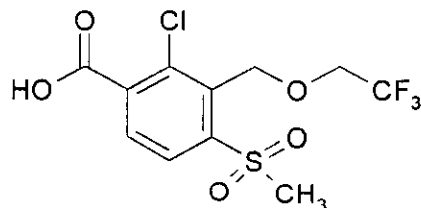


Code name: AE 0172747-trifluoroethoxymethyl-d4
 Chemical name (CAS): 2-{2-chloro-4-mesyl-3-[(2,2,2-trifluoroethoxy-1,1-d2)-methyl-d2]-benzoyl}-1,3-cyclohexanedione
 Empirical formula: C₁₇ H₁₂ Cl D₄ F₃ O₆ S
 Molecular weight: 444.84 g/mol

Reference standard:
 Standard no.: K-1418
 Reference no.: 2003BRP003-215
 Origin: Bayer CropScience, Stilwell, KS, USA

AE 0456148:

Structural formula:

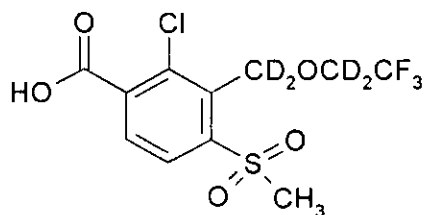


Chemical code: AE 0456148
 Chemical name (CAS): 2-chloro-4-mesyl-3-[(2,2,2-trifluoroethoxy)methyl]-benzoic acid
 Empirical formula: C₁₁ H₁₀ Cl F₃ O₅ S
 Molecular weight: 346.71 g/mol

Reference standard:
 Certificate of analysis: AZ 10576
 Purity: 93.8%
 Expiry date: April 2006
 Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt, D-65926 Frankfurt am Main, Germany

AE 0456148-trifluoroethoxymethyl-d4 (used as internal standard for the test item AE 0456148):

Structural formula:



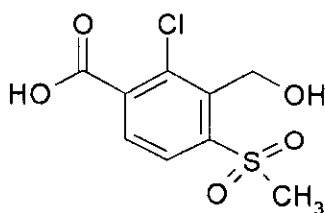
Code name: Free acid-trifluoroethoxymethyl-d4,
AE 0172747-acid-trifluoroethoxymethyl-d4
Chemical name (CAS): 2-chloro-4-(methylsulfonyl)-3-[(2,2,2-trifluoroethoxy-1,1-d2)methyl-d2]benzoic acid
Empirical formula: C₁₁ H₆ Cl D₄ F₃ O₅ S
Molecular weight: 350.73 g/mol

Reference standard:

Standard no.: K-1229
Purity: 98.7%
Expiry date: June 2008
Origin: Bayer CropScience, Stilwell, KS, USA

AE 1392936:

Structural formula:



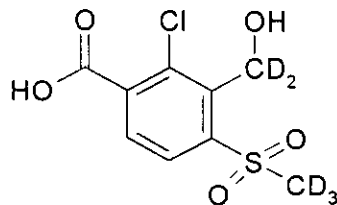
Chemical code: AE 1392936
Chemical name (CAS): 2-chloro-3-hydroxymethyl-4-mesylbenzoic acid
Empirical formula: C₉ H₉ Cl O₅ S
Molecular weight: 264.68 g/mol

Reference standard:

Certificate of analysis: AZ 12786 (and AZ 10910)
Purity: 93%
Expiry date: July 2008 (July 2005)
Origin: Bayer CropScience GmbH, PT – Analytics Frankfurt,
D-65926 Frankfurt am Main, Germany

AE 1392936-benzyl-methylsulfonyl-d5 (used as internal standard for the test item AE 1392936):

Structural formula:



Code name: AE 0172747 P-2-benzyl-methylsulfonyl-d5
 Chemical name (CAS): 2-chloro-3-(hydroxymethyl-d₂)-4-(methyl-d₃-sulfonyl)-benzoic acid
 Empirical formula: C₉H₄ClD₅O₅S
 Molecular weight: 269.71 g/mol

Reference standard:

Standard no.: K-1239
 Purity: 91.8%
 Expiry date: October 2008
 Origin: Bayer CropScience, Stilwell, KS, USA

3.2 Test System

The method was validated using *River Rhine water* 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 LEV UMG, Building W15. Water types are summarised in Table 1.

Complete water parameters are reported in Table 12 and Table 13.

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: AE-005-W05-02
 Title of the method: Method of Analysis for the Determination of Residues of AE 0172747 and its Metabolites AE 0456148 and AE 1392936 in Water Using LC/MS/MS – Revision W05-02.
 Author of the method: Robert J. Seymour, Ph.D.
 Diane Beck
 Bayer CropScience LP
 17745 S Metcalf Avenue
 Stilwell, Kansas 66085
 Reference: Method AE-005-W05-02
 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 0.05 µg/L	Level 0.25 µg/L
River Rhine	2	5	5
Tap Water Monheim	2	5	5

Additionally, a solvent blank without internal standards added was analysed.

4.1.1 Outline of the Method

Water Samples were brought to room temperature and mixed completely before removing a sub-sample for analysis. Volumes of 10 mL were removed to suitable glass test tubes with standardised grinding and 250 µL of formic acid were added. 100 µL of an appropriate internal standard was given to each sample. Appropriate volumes of fortification solution were added to the LOQ and 5x LOQ control samples. The glass test tubes were capped and shaken to mix. Approximately 1 to 1.5 mL of the samples were transferred to an autosampler vial and capped.

Control samples were treated in the same manner, only the fortification with the fortification standard was excepted.

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: Applied Biosystems API 4000 LC/MS/MS System
with Valco Divert Valve and Turbo Ion Electrospray interface
64293 Darmstadt, Germany

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): SymmetryShield RP8 5 µm, length 150 mm, i.d. 3 mm
Order No. WAT094243
Waters GmbH
65760 Eschborn, Germany

Acetonitrile: for HPLC, super gradient grade
Riedel de Haën, No. 34998
30926 Seelze, Germany

Acetic acid (100%): p.a.
Merck, No.1.00063.1011
64271 Darmstadt, Germany

Formic acid (98 - 100%): purest
Riedel-de Haën, No.27001
30926 Seelze, 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 modified from those described in Appendix 1 of the original method report AE-005-W05-02 (see Table 3 to Table 5).

Table 3: Modified Liquid Chromatographic Conditions

Column:*	SymmetryShield™ RP 8, 150 x 3 mm
Particle size:	5 µm
Oven temperature:	40 °C
Injection volume:	90 µL
Flow rate:*	0.75 mL/min.
Run time:*	15 minutes
Mobile phase:	A: milli-Q-water / acetic acid (985/15, v/v/v) B: acetonitrile
Retention times:*	approx. 3.4 min for AE 1392936 approx. 8.7 min for AE 0172747 approx. 9.5 min for AE 0456148

Table 4: HPLC Gradient*

Time [min]	A [%]	B [%]
0.0	90	10
3.0	90	10
8.0	10	90
10.0	10	90
10.1	90	10
15.0	90	10

Table 5: Valco Valve Method Properties*

Time [min]	
2.5	switch eluent stream into interface
10.0	switch eluent stream into waste

* parameters deviating from the original method

MS/MS parameter settings were optimized for the instrument being used and therefore not identical with those reported in method AE-005-W05-02.

To enhance the sensitivity for the determination of AE 1392936, the MS/MS-detection was split into two periods, the first period for measurement of AE 1392936 (dwell time: 800 msec for AE 1392936 and 200 msec for AE 1392936-d₅), the second period for the determination of AE 0172747 (dwell time: 100 msec for AE 0172747 and 100 msec for AE 0172747-d₄) and AE 0456148 (dwell time: 400 msec for AE 0456148 and 400 msec for AE 0456148-d₄). These parameters ensured optimal results of measurements for all compounds.

Table 6: Acquisition Parameters

<u>Period</u>	<u>Scan Type</u>	<u>Polarity</u>	<u>Resolution Q1</u>	<u>Resolution Q3</u>
1	MRM	Negative	Unit	Low
2	MRM	Negative	Unit	Unit

<u>Analyte</u>	<u>Period</u>	<u>Q1 Mass (amu)</u>	<u>Q3 Mass (amu)</u>	<u>Dwell (msec)</u>	<u>Parameter</u>	<u>Value</u>
AE 1392936	1	262.90	189.00	800	DP	-45.00
					EP	-10.00
					CE	-16.00
					CXP	-11.00
AE 1392936-d ₅	1	267.90	192.00	200	DP	-45.00
					EP	-10.00
					CE	-16.00
					CXP	-11.00
AE 0172747	2	439.03	403.00	100	DP	-35.00
					EP	-10.00
					CE	-18.00
					CXP	-17.00
AE 0172747-d ₄	2	443.00	407.00	100	DP	-35.00
					EP	-10.00
					CE	-18.00
					CXP	-17.00
AE 0456148	2	344.91	217.10	400	DP	-30.00
					EP	-10.00
					CE	-18.00
					CXP	-15.00
AE 0456148-d ₄	2	348.90	221.10	400	DP	-30.00
					EP	-10.00
					CE	-18.00
					CXP	-15.00

<u>Parameter</u>	<u>Value</u>
CUR	50.00
GS1	50.00
GS2	90.00
IS	-4200.00
TEM	500°C
CAD	6.00

4.1.5 Calculation

Calculations were performed using the computer software MS-EXCEL. In general, the program uses nine decimal places for calculations. The results given are rounded values. Thus, rounding "errors" may occur if recalculations are made using the listed figures.

4.1.5.1 Calculation of AE 0172747, AE 0456148 and AE 1392936 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 area ratio (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

4.1.5.2 Calculation of Recoveries

1. Calculate the mean concentration in the recovery sample according to 4.1.5.1.
2. Calculate the percent recovery as follows:

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

Recovery: Recovered concentration of analyte in % found in the fortified sample
 Mean residue: Mean residue in the fortified sample in µg/L determined according to 4.1.5.1
 Fortification level: Fortified concentration of analyte in µg/L

4.1.6 Deviations from the Method

Within the analytical procedure for determination of AE 0172747 and its metabolites AE 0456148 and AE 1392936 several details have been changed.

Since the HPLC system used for the ILV was not optimized for working with low flow rates, the HPLC parameters had to be adjusted. According to method AE-005-W05-02 two columns **SymmetryShield RP8 (50 mm x 2.1 mm)** in series had been used. Within the ILV procedure a column **SymmetryShield RP8 (150 mm x 3 mm)** was used, due the other columns could not reflected the reported retention times and resolution. The **flow rate** was increased from 0.2 mL/min. to 0.75 mL/min. and the **gradient program** was modified.

To enhance the sensitivity for the determination of AE 1392936, the MS/MS-detection was split into two periods, the first for measurement of AE 1392936, the second for the determination of AE 0172747 and AE 0456148.
 All changed LC/MS/MS parameters used during this study are given in section 4.1.

5.2 Linearity of the Detector

The linearity of the detector response for AE 0172747 and its metabolites AE 0456148 and AE 1392936 were tested by injections of standard solutions. The following concentrations were measured:

Table 7: Standard Concentrations for the Determination of Detector Linearity

Substance	Concentration [$\mu\text{g/L}$]				
AE 0172747	0.02	0.05	0.1	0.25	0.5
AE 0456148	0.02	0.05	0.1	0.25	0.5
AE 1392936	0.02	0.05	0.1	0.25	0.5