

STUDY TITLE

Method Validation Study for the Determination of Residues of Florasulam and its 5-OH Metabolite in Surface Water, Ground Water and Drinking Water by Liquid Chromatography with Tandem Mass Spectrometry

DATA REQUIREMENTS

European Council Directive 91/414/EEC, as amended by  
European Commission Directive 96/46/EC.  
European Commission Guidance Documents on Residue Analytical

## INTRODUCTION

Analytical method GRM 07.23, “Determination of Residues of Florasulam and its 5-OH Florasulam Metabolite in Waters by Liquid Chromatography with Tandem Mass Spectrometry Detection”, was developed and validated at Dow AgroSciences LLC.

In the present study the method was revised for direct injection of water samples into the LC/MS/MS system and found to be suitable for the determination of residues of florasulam and its 5-OH florasulam metabolite in waters over the concentration range of 0.05-0.5 µg/L. The validated limit of quantitation of the method was 0.05 µg/L.

## ANALYTICAL

### Preparation and Storage of Samples

#### *Drinking (Tap) Water*

Water was collected from a PTRL Europe laboratory tap located in Ulm, in Southern Germany. The appearance of the water was clear and without any odor. The water was characterized for physical and chemical properties as follows: pH 8.36, total water hardness: 2.40 mmol/L (Deutsche Härtegrade, 13.5°d). The water was characterized by accredited Institute Alpha (Ulm, Germany following common DIN or EN guidelines and methods, non-GLP).

#### *Surface (River) Water*

Water was collected on 23-Aug-10 from the Brenz River in Herbrechtingen, located in Southern Germany. The water was characterized for physical and chemical properties as follows: pH 8.43. The river water was characterized by accredited Institute Alpha (Ulm, Germany following common DIN or EN guidelines and methods), resulting in the following (non-GLP):

total water hardness:	2.98 mmol/L (Deutsche Härtegrade, 16.7°d)
TOC (total organic carbon, EN 1484:1997):	0.84 mg/L
DOC (diluted organic carbon, EN 1484: 1997):	0.64 mg/L
Silt content (EN 872 Whatman GF 6):	<5.0 mg/L
Turbidity (EN 7027:1999)	0.64 NTU

#### *Ground (Well) Water*

Water was collected on 23-Aug-10 from Herbrechtingen, in Southern Germany. The water was characterized for physical and chemical properties as follows: pH 8.28. The ground water was

characterized by accredited Institute Alpha (Ulm, Germany following common DIN or EN guidelines and methods), resulting in the following (non-GLP):

total water hardness:	3.37 mmol/L (Deutsche Härtegrade, 18.9°d)
TOC (total organic carbon, EN 1484:1997):	1.1 mg/L
DOC (diluted organic carbon, EN 1484: 1997):	1.0 mg/L
Silt content (EN 872 Whatman GF 6):	<5.0 mg/L
Turbidity (EN 7027:1999)	5.80 NTU

Water was stored at room temperature in the dark when not used.

### Apparatus

Analytical balance: Sartorius RC210D (used for analytical standard).

Ultrasonic bath: Transsonic 460 (Elma).

Vortex mixer Assistent Reamix.

Typical glassware and laboratory equipment.

All the glassware was cleaned in a laboratory dishwasher and air-dried before use.

### Reagents, Chemicals and Miscellaneous

Acetonitrile, acetone, methanol, all HPLC or residue grade (Promochem).

Millipore water (supply at PTRL).

Glacial acetic acid (100 %, Merck).

### LC-MS/MS System

Agilent 1200 Series HPLC system (vacuum solvent degasser, binary HPLC pump, column oven), and CTC Analytics HTC-Pal Autosampler.

Supelco Ascentis Express 100 x 3 mm, 2.7- $\mu$ m particle size with Phenomenex C<sub>18</sub>, 4 x 3 mm, pre-column.

Applied Biosystems MDS Sciex API 5500 triple quadrupole LC-MS/MS system with TurboIonSpray ESI source. Analyst 1.5 Instrument control and data acquisition software.

### Preparation of Solutions and Standards

A stock solution of florasulam and its 5-OH metabolite were prepared as follows:

Analyte	Purity [%]	Amount weighed [mg]	Dilute to [mL]	Concentration of stock solution [mg/mL]
Florasulam	99.7	20.06	20 (Acetone)	1.0
5-OH Florasulam	98.1	20.36	20 (Acetonitrile)	1.0

These solutions were further diluted volumetrically into acetonitrile to obtain mixed intermediate solutions with 10 and 0.10 µg/mL per analyte. These intermediate solutions were further diluted volumetrically into acetonitrile to obtain mixed fortification solutions with 5.0 and 0.50 ng/mL per analyte.

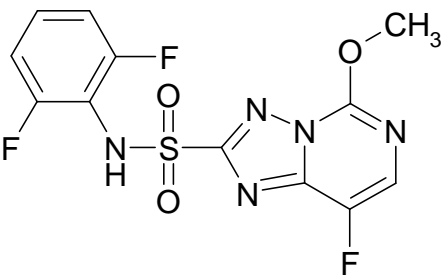
Calibration solutions were prepared by volumetric dilution to obtain concentrations of 1.0 µg/mL and 0.010 µg/mL (intermediate), and further to obtain concentrations of 1.0, 0.50, 0.20, 0.10, 0.050, 0.025 and 0.010 ng/mL in acetonitrile/water; 10/90; v/v used for calibrations.

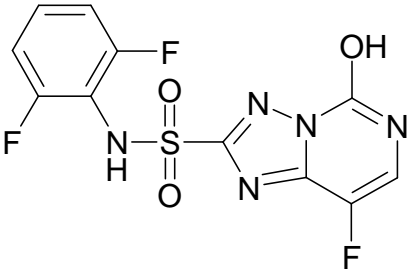
#### Stability of Solutions and Extracts

All solutions were stored in a refrigerator (at approximately ≤ 8 °C) when not in use.

Final sample dilutions ready for analysis were stable up to nine days as shown by acceptable recovery results, when re-analyzing selected extracts after they had been stored refrigerated for 5 to 9 days.

The following analytical standards (obtained from the Sponsor) were utilized during the method validation:

Common Name of Compound	Structure and CAS Name
Florasulam  Molecular Formula: C <sub>12</sub> H <sub>8</sub> F <sub>3</sub> N <sub>5</sub> O <sub>3</sub> S Formula Weight 359.29 Nominal Mass: 359  CAS Number: 145701-23-1	 <p><i>N</i>-(2,6-difluorophenyl)-8-fluoro-5-methoxy[1,2,4]triazolo[1,5-<i>c</i>]pyrimidine-2-sulfonamide</p>

5-OH Florasulam		 <p><i>N</i>-(2,6-difluorophenyl)-8-fluoro-5-hydroxy[1,2,4]triazolo[1,5-<i>c</i>]pyrimidine-2-sulfonamide</p>		
Molecular Formula: C <sub>11</sub> H <sub>6</sub> F <sub>3</sub> N <sub>5</sub> O <sub>3</sub> S				
Formula Weight: 345.269				
Nominal Mass: 345				
CAS Number: N/A				
Test Substance/ Analytical Standard	AGR/TSN Number	Percent Purity	Certification Date	Reference
Florasulam	TSN100381	99.7%	02-May-2008 Recertification: 25-Apr-2012	FAPC08-163723
5-OH Florasulam	TSN101151	98.1%	30-Jun-2009 Recertification: 19-Jun-2013	FAPC 09-225597

### Preparation of Water Samples for LC/MS/MS Injection

1. An aliquot of 1.0 mL ( $V_{\text{Sample}}$ ) water was dosed into an autosampler vial.
2. 100  $\mu\text{L}$  of the corresponding fortification solution were added, if necessary.
3. 100  $\mu\text{L}$  of acetonitrile were added to blank control samples.
4. Shake with vortexer.
5. Determination of the analytes was done by using LC/MS/MS.

### Analytical Instrumentation and Equipment

For Q1 and product ion scan of florasulam and its 5-OH metabolite see Figure 18 and Figure 19.

The following LC/MS/MS method was used:

Liquid Chromatography Operating Conditions

Instrumentation:	CTC Analytics HTC PAL Autosampler Agilent Model 1200 binary pump Agilent Model 1200 degasser		
Column:	Supelco Ascentis Express 100 x 3 mm, 2.7- $\mu$ m particle size (C18-type stationary phase) Securityguard: Phenomenex, C18, 4 x 3 mm,		
Column Temperature:	40°C		
Injection Volume:	60 $\mu$ L		
Mobile Phase:	A – water with 0.1% acetic acid B – acetonitrile/methanol (1/1, v/v) with 0.1% acetic acid		
Flow Rate:	500 $\mu$ L/min		
Gradient:	Time, min	A, %	B, %
	0.00	90	10
	1.00	90	10
	5.00	0	100
	9.00	0	100
	9.10	90	10
	13.00	90	10

Mass Spectrometry Operating Conditions

Instrumentation:	Applied Biosystems API 5500 LC/MS/MS System Applied Biosystems Analyst 1.5 data system			
Interface:	TurboIonSpray			
Scan Type:	MRM			
Resolution:	Q1 – Unit, Q3 – Unit			
Curtain Gas (CUR):	30			
Collision Gas (CAD):	Medium			
GS1:	40			
GS2:	60			
Temperature (TEM):	450°C			
Polarity:	Negative			
IonSpray Voltage (IS):	-4500 V			
Declustering Potential (DP):	-95			
Entrance Potential (EP):	-10			
Analytes:	Ion, m/z		Dwell Time, ms	CE/CXP, V
	Q1	Q3		
Florasulam (quantitation)	358.2	167.0	200	-24/-5
Florasulam (confirmation)	358.2	152.1	300	-44/-5
5-OH Florasulam (quantitation)	344.1	324.1	200	-24/-5
5-OH Florasulam (confirmation)	344.1	104.0	300	-40/-5

## Sample Analysis

For analysis of florasulam and its 5-OH Metabolite by LC-MS/MS, calibration functions were established by injecting calibration solutions in neat solvent (acetonitrile/water; 10/90; v/v) with at least 5 different concentration levels in a range from 0.010 to 1.0 ng/mL. Calibration functions were calculated by linear regression calculation, applying "1/x" weighting. Correlation coefficients "r" for all calibration curves were always > 0.99.

Representative calibration curves with calibration functions are shown in Figure 1 through Figure 3, including peak areas and confirmatory peak area ratios, and a plot showing response factors versus concentration. LC-MS/MS ion chromatograms of calibration solutions with the lowest calibration level are depicted in Figure 4 and Figure 5.

LC-MS/MS ion chromatograms of water samples are presented in Figure 6 through Figure 17.

Repeatability of LC-MS/MS determination was demonstrated by duplicate injections of at least one extract per fortification level and set.

## Calculations

Quantification of the analytes were accomplished by the external standard method. Results derived from LC-MS/MS analysis and calculations are shown in detail in Table 1 to Table 3. The values reported in the tables are calculated with full precision, but are displayed with rounding.

The concentrations  $C_{\text{End}}$  (ng/mL) of the analytes found in the final dilution prepared for analysis ( $V_{\text{End}} = 1.1$  mL) were converted to the residues  $R$  ( $\mu\text{g/L}$ ) of the analytes in the original water sample aliquot ( $V_{\text{Water}} = 1.0$  mL) as follows:

$$\begin{aligned} R &= C_{\text{End}} \times (V_{\text{End}} / V_{\text{Sample}}) \\ &= C_{\text{End}} \times \text{Multiplier M} \\ &= C_{\text{End}} \times 1.10 \end{aligned}$$

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$$\text{Rec.} = (R / R_{\text{fortified}}) \times 100 \%$$

### *Example for Calculation*

The calculation is exemplified with the tap water specimen PTRL-ID P2292-34.

1.0 mL ( $V_{\text{Water}}$ ) of water were fortified at 0.5  $\mu\text{g/L}$  (10xLOQ) with florasulam by dosing 0.10 mL of the 5.0 ng/mL fortification solution to obtain a final volume  $V_{\text{End}} = 1.1$  mL.

The final extract was examined by LC/MS/MS in run file P2292-051, resulting in a florasulam concentrations  $C_{\text{End}}$  of 0.462 ng/mL (358 m/z  $\rightarrow$  167 m/z), and a residue result R of 0.508  $\mu\text{g/L}$ .

Recoveries (Rec.) were calculated for the fortified specimens as follows:

$$\begin{aligned}\text{Rec.} &= (R / R_{\text{fortified}}) \times 100 \% \\ &= (0.508 \mu\text{g/L} / 0.50 \mu\text{g/L}) \times 100 \% \\ &= 102 \%\end{aligned}$$

### Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the “AVERAGE” function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level of one matrix type was calculated using the “STDEV” function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % RSD, was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

### Confirmatory Evaluation

The presence of each analyte is confirmed by comparing the liquid chromatography retention times of the analytes in the calibration standards with those found in the samples when monitoring two characteristic MS/MS transitions.