INTRODUCTION

Analytical LLC Method ERC 96.21, "Determination of Residues of XDE-570 and 5-hydroxy XDE-570 in Soil Using Organic Extraction" (Appendix B), was developed and validated at Dow AgroSciences LLC. The method was found to be suitable for the determination of residues of florasulam and its 5-OH metabolite in soil over the concentration range of 0.050-50.0 µg/kg. The validated limit of quantitation of the method was 0.050 µg/kg.

An independent laboratory validation (ILV) of LLC Method ERC 96.21 was conducted on one sandy loam and one clay loam soil type in order to satisfy the requirements of the Subdivison N (Environmental Fate), Series 164-1; Publication of Addenda for Data Reporting E, K, and N Requirements for Pesticide Assessment Guidelines; Guideline OPPTS 850.7100 "Public Draft"; PR Notices 96-1 and 86-5 and EU Council Directive 91/414/EEC, SANCO/825/00 rev. 7.

The independent laboratory, the Study Director, and the analysts chosen to conduct the ILV were unfamiliar with the method, both in its development and subsequent use in analyzing samples. The independent laboratory used all of its own equipment and supplies, so that there was no common link between Dow AgroSciences and the ILV analysts. Throughout the conduct of the study, any communications between Dow AgroSciences and the Study Director and/or the analyst were logged for inclusion in the report. No one from Dow AgroSciences was allowed to visit the independent laboratory during the ILV trial to observe, offer help, or assist the chemists or technicians. These steps successfully maintained the integrity of the ILV study.

ANALYTICAL

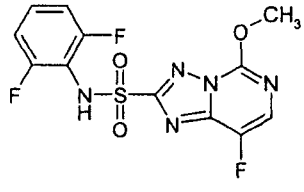
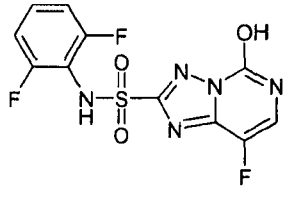
Preparation and Storage of Samples

Two European standard soils (one sandy loam and one clay loam) were obtained from LUFA Speyer[1] sieved (2 mm). Soil characterization information is given in Appendix D.

Preparation of Solutions and Standards

Materials used (obtained e.g. from Merck, Fluka, Sigma-Aldrich, Riedel de Haën, Varian, J.T. Baker and Promochem) were of equivalent specifications as described in Section 5 of method ERC 96.21. Reagents were prepared as given in Appendix B in Section 5.2 of method ERC 96.21.

The following analytical test substances/analytical standards were obtained by the sponsor and were utilized during the independent laboratory method validation:

Common Name of Compound	Structure and CAS Name
Florasulam Molecular Formula: $C_{12}H_8F_3N_5O_3S$ Formula Weight 359.29 Nominal Mass: 359 CAS Number: 145701-23-1	 *N*-(2,6-difluorophenyl)-8-fluoro-5-methoxy[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide
5-OH Florasulam Molecular Formula: $C_{11}H_6F_3N_5O_3S$ Formula Weight 345.269 Nominal Mass: 345 CAS Number: N/A	 *N*-(2,6-difluorophenyl)-8-fluoro-5-hydroxy[1,2,4]triazolo[1,5-*c*]pyrimidine-2-sulfonamide

[1] Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, D-67346 Speyer.

Test Substance/ Analytical Standard	AGR/TSN Number	Percent Purity	Certification Date	Reference
Florasulam	TSN100381	99.7%	02-May-2008	FAPC08-163723
5-OH florasulam	TSN101151	98.1%	21-Jun-2005	FA&PC 053132

Stock and fortification solutions were prepared as described in Section 6.1 of method ERC 96.21 (Appendix B). Calibration standard solutions were prepared as described in Section 6.2 of method ERC 96.21.

The identity and structures of florasulam (XDE-570) and its 5-OH metabolite are given in Appendix C.

Fortification of Recovery Samples

Two ILV trials of the method were conducted and consisted for each soil type:

1 reagent blank (containing no matrix or analyte)

2 unfortified control samples

5 control samples fortified at 0.050 µg/kg (the LOQ of the method)

5 control samples fortified at 0.50 µg/kg (10 x LOQ).

Sample Extraction, Purification and Analysis

The ILV trial was conducted as described in Section 6.6 of method ERC 96.21 (Appendix B), with some modifications:

Sample analysis was performed as described in Section 6.6 up to Section 6.6.3 (elution from PolarPlus C18 cartridge). The additional clean-up steps described in Sections 6.6.4 to 6.6.6 (SAX Bond Elut SPE clean-up) were only used in the 1st ILV set with sandy loam, resulting in total loss of the analytes[2], thus this SAX SPE clean-up was omitted in the subsequent ILV trials and only the 1st C_{18} clean-up procedure was performed.

[2] In a 1st ILV trial performed with the sandy loam no analytes were detected in the final extracts obtained after elution of the SAX Bond Elut column with 15 mL of 0.1 M HCl/methanol (9/1 v/v), as detailed in section 6.6.4 of the original method.
Assessing the SAX SPE clean-up in separate experiments indicated that the methanol portion has to be increased to elute the analytes with e.g. 15 mL of 0.1 M HCl/methanol (1/1 v/v) for significant recovery.

As described in Section 6.6.3 analytes were eluted from the C18 material using 3.0 mL acetonitrile. Thereof a 1.5 mL aliquot was transferred in a silanised autosampler vial, reduced to the aqueous residue of approx. 200 µL in a gentle stream of nitrogen. The residue was filled up with 1 % aqueous acetic acid to 1.0 mL, re-dissolved with the aid of sonication and submitted for HPLC/MS/MS analysis.

Analytical Instrumentation and Equipment

Prior to initiation of the first ILV trial, the independent laboratory conducted preliminary studies necessary for establishing acceptable performance of the chromatographic instrumentation to be used.

These preliminary studies included establishing that adequate HPLC retention times of the analytes and MS/MS detector sensitivity in the positive mode (as used in the original method validation) as well as in the negative mode, which was expected to provide better sensitivity and selectivity (by adding a 2nd mass transition for confirmation) and to be less prone to matrix effects (also see Appendix A, which demonstrates matrix suppression for 5-OH florasulam when detected in the positive mode, but acceptable recoveries when detected in the negative mode).

The instrumental conditions used during the 2nd and successful ILV trials were similar to the conditions described in Section 6.3 of method ERC 96.21 (see Appendix B), with adaptations as given below:

Liquid Chromatography Operating Conditions

Instrumentation:	CTC Analytics HTC PAL Autosampler Agilent Model 1200 binary pump Agilent Model 1200 degasser
Column:	Phenomenex Luna C_{18} 250 x 4.6 mm, 5-µm particle size Securityguard: Phenomenex, C_{18}, 4 x 3 mm
Column Temperature:	30°C
Injection Volume:	80 µL
Mobile Phase:	A – water/acetonitrile/acetic acid (60/40/1,v/v/v) B – acetonitrile with 1% acetic acid
Flow Rate:	800 µL/min

Gradient:

Time, min	A, %	B, %
0.00	90	10
10.00	90	10
11.00	0	100
15.00	0	100
16.00	90	10
21.00	90	10

Mass Spectrometry Operating Conditions

Instrumentation:	Applied Biosystems API 4000 LC/MS/MS System Applied Biosystems Analyst 1.4.2 data system
Interface:	TurboIonSpray
Scan Type:	MRM
Resolution:	Q1 – Unit, Q3 – Unit
Nebulizer Gas (GS1)	40
Turbo Gas (GS2)	70
Curtain Gas (CUR):	20
Collision Gas (CAD):	5
Temperature (TEM):	500°C
Polarity:	Negative
IonSpray Voltage (IS):	-4500 V
Declustering Potential (DP):	-65
Entrance Potential (EP):	-10

Analytes:	Ion, m/z		Dwell Time, ms	CE/CXP, V
	Q1	Q3		
florasulam (quantitation)	358.1	166.9	200	-22/-11
florasulam (confirmation)	358.1	152	200	-48/-13
5-OH florasulam (quantitation)	344.0	324	200	-24/-9
5-OH florasulam (confirmation)	344.0	104	200	-42/-7

Verification that the untreated control sample was free from any interferences was not performed prior to initiation of the 1st ILV trial.

Calculations

Typical calibration curves using both the quantitative and the confirmatory ion transitions for the determination of florasulam and its 5-OH florasulam metabolite in soil extracts are presented in Figure 1 and Figure 2, respectively. Linear regression calculation was performed by the Analyst software, with 1/x weighting, using the residue concentration, in ng/mL, for the X-axis, versus the analyte peak area for the Y-axis.

Typical chromatograms generated using both the quantitative and the confirmatory ion transitions are presented in Figure 3 and Figure 4 (calibration solutions) and in Figure 5 to Figure 16 (soil samples).

For the calculation of residues the following formula was used:

$R = c_{End} \times (V_{Ex} \times V_{End}) / (V_1 \times W)$

$R = c_{End} \times$ Multiplier M

Where :

R: Analyte residue in µg/kg or ppb.

c_{End}: Final concentration of analyte in extract in ng/mL.
(where multiple injections were evaluated: mean).

V_{Ex}: Extract volume after C18 SPE cartridge: 3.0 mL

V_1: Aliquot of extract used for LC/MS/MS: 1.5 mL.

V_{End}: Volume of final extract used for LC/MS/MS: 1.0 mL.

W: Specimen weight: 25 g.

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

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

Example for florasulam:

The calculation is exemplified with the sandy loam specimen (PTRL-ID P1485-55) for which W = 25 g were fortified at 10xLOQ (0.5 µg/kg) and extracted. After the 1st C18 SPE cartridge clean-up the analytes are eluted with V_{Ex} = 3.0 mL, thereof V_1 = 1.5 mL were taken, evaporated to the aqueous residue and re-dissolved in V_{End} = 1.0 mL.

The final extract was examined by LC/MS/MS in run file P1485-177, resulting in a florasulam concentration c_{End} of 4.81 ng/mL.

Thus:

$R = c_{End} \times (V_{Ex} \times V_{End}) / (V_1 \times W)$

$= 4.81 \text{ ng/mL} \times (3 \text{ mL} \times 1 \text{ mL}) / 1.5 \text{ mL} \times 25 \text{ g})$

$= 4.81 \text{ ng/mL} \times 0.08 \text{ mL/g}$

$= 0.38 \text{ ng/g (µg/kg)}$

Recovery: 77 %

The florasulam values were calculated using a computerized spreadsheet that used more decimal places than those displayed. As a result, the calculated values shown in this example may vary slightly if the values are recalculated using the displayed parameters.

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. Statistical outliers were eliminated using the Dixon Test, where the statistical experimental Q-value is compared with defined critical Q-values using a confidence level of 99 %.

Confirmatory Evaluation

The presence of florasulam and its 5-OH metabolite is confirmed by comparing the liquid chromatography retention times of the analytes in the calibration standards with those found in the samples and by monitoring two characteristic MS/MS transitions per analyte.

Problems Encountered, Changes or Modifications Made, and Critical Steps

The following problems or critical steps were encountered with the methodology.

The extraction steps described in Sections 6.6.4 to 6.6.6 were omitted. The 1st ILV trial for sandy loam was not successful when performed exactly as described in these sections. There was complete retention of both analytes on the second SPE clean-up cartridge (the SAX SPE cartridge). Different elution profiles tested showed that both analytes only elute when using a composition of 0.01 M HCl/methanol of 1/1 instead of 9/1 (v/v) as described in the method. By using the 0.01 M HCl/methanol of 1/1 to elute the analytes from the SAX cartridge, the ethyl acetate liquid/liquid partition step as described in Section 6.6.5 of the method was no longer practical.

Therefore only the 1st C_{18} SPE clean-up procedure was performed. As described in Section 6.6.3

analytes were eluted using 3.0 mL acetonitrile. Thereof a 1.5 mL aliquot was transferred in a silanised autosampler vial, reduced to the aqueous residue under a gentle stream of nitrogen. The residue was re-dissolved with the aid of sonication in a total volume of 1 mL of 1 % aqueous acetic acid and submitted to the HPLC/MS/MS analysis.

The LC/MS/MS determination of florasulam and its 5-OH metabolite was performed using negative-ion electrospray ionization due to signal suppressions observed in the positive ionization mode.
It is recommended, that the final extracts are injected without delay on the HPLC/MS/MS system due to possible instability of the analytes.

Sample Analysis Time Requirements

One set of 13 samples required approximately 8 hours work to complete in the laboratory, followed by unattended over-night LC/MS/MS analysis, followed by approximately 2 hours of evaluation and data transcription. Thus, a complete sample set (consisting of 13 samples) can be completed in approximately one and one half calendar days.

Communications

E-mail contacts between the Study Director at the independent laboratory and the study monitor on 30-Jun-08 and 11-Jul-08 reported and discussed results obtained for the 1st ILV trials and concluded that 2nd ILV trials should be performed using the modifications of analytical method ERC 96.21 reported herein.
No contacts with the method developer or others familiar with the method were necessary.