ANALYTICAL

Preparation and Storage of Samples

The independent laboratory validation was carried out on two soil specimens. The untreated control soil specimens were sourced directly by CEMAS.

On arrival, unique sample numbers were assigned to the soil samples to track them during receipt, storage and analysis. The prepared specimens were placed in a freezer set to maintain a specimen temperature of ≤-18°C where they were retained at all times unless removed for analysis. Full sample details are included in the raw data package.

Specimen Identifier	Specimen Type	Origin
CCON/052/001	Soil (silty clay loam)	Transferred from CEMAS study
CCON/053/001	Soil (clay loam)	Transferred from CEMAS study

The soil specimens were characterised at CEMAS; Study Number CEMS-4013. Details of the GLP characterisation results are as follows:

GLP Characterisation	CCON/052/001	CCON/053/001
Soil pH (H_2O)	6.4	7.6
Soil pH (0.01M $CaCl_2$)	5.9	7.2
Organic Matter (Walkley-Black) (% w/w)	2.1	2.2
Organic Carbon (Walkley-Black) (% w/w)	1.2	1.3
Sand Fraction (2.00 – 0.063 mm) (% w/w)	3	40
Silt Fraction (0.063 – 0.002 mm) (% w/w)	76	34
Clay Fraction (<0.002 mm) (% w/w)	21	26
Textural Classification (UK)	Silty Clay Loam	Clay Loam
Cation Exchange Capacity (Na-Saturation at pH 7.00) (meq/100g)	11.45	12.9
Soil Bulk Density – intact core (g/mL)	1.44	1.47
Microbial Biomass (Cmic)	325.4 mg/kg	429.4 mg/kg
Microbial Biomass (as % TOC)	2.50	2.86

Preparation of Solutions and Standards

Reagents used were of equivalent specifications to those described in the analytical method. The following analytical test substances/analytical standards were utilized during the independent laboratory method validation:

Test Substance/Analytical Standard:	Ethalfluralin
Supplier:	Dow AgroSciences (Sponsor)
Test Substance No.	TSN101281
Batch/Lot no:	597-C049-003
Purity:	99.8%
Expiry date:	08 January 2013
Storage:	Coldroom

Standard solutions and calibration standard solutions were prepared as described in the analytical method. Full details of these materials are included in the raw data package for the study along with details of the preparation of all analytical and fortification standards prepared from the primary reference items. The test/reference item will be retained until expiry and then disposed of. The samples may be disposed of following completion of the Study (issuance of the Final Report) with the approval of the Study Monitor.

Fortification of Recovery Samples

The control specimens were fortified as described below:

Matrix	Reference Item	Untreated Control Specimens	Replicates at Fortification Level (LOD)*	Replicates at Fortification Level (LOQ)**	Replicates at Fortification Level (10xLOQ)
Soil (silty clay loam)	Ethalfluralin	2	1 at 0.003 mg/kg	5 at 0.01 mg/kg	5 at 0.1 mg/kg
Soil (clay loam)	Ethalfluralin	2	1 at 0.003 mg/kg	5 at 0.01 mg/kg	5 at 0.1 mg/kg

*LOD – Limit of determination
**LOQ – Limit of quantitation

Ten grams of the control soil specimen were measured into individual 4 oz jars. Each sample was fortified as per the table above. One sample was fortified to achieve the fortification level 0.003 mg/kg (LOD), five samples were fortified at 0.01 mg/kg (LOQ) and five samples were fortified to achieve the upper fortification levels of 0.1 mg/kg. The fortification solution was injected directly onto the matrix.

Sample Extraction, Purification and Analysis

Specimens were assayed according to the analytical method stated in the Study Plan CEMS-5394. (Appendix A).

In summary, 20 mL of an acetonitrile/water (99:1) solution was added to each of the samples. Samples were shaken and centrifuged. The final solutions were diluted and purified in a SPE experiment using a 300-mg multi mode (C18, SAX, SCX) solid-phase extraction column. Ethalfluralin was eluted from the SPE column with hexane. Three mg of peanut oil was added to the samples and the volume was brought up to 5 mL with hexane . The final samples were analyzed by gas chromatography with electron impact mass spectrometry (GC-MS).

Full extraction details:

Sample Analysis of Ethalfluralin

Ten ±0.05 gram portions of each soil matrix were measured into individual 4 oz jars. For recovery samples, an appropriate volume of the spiking solutions was added to obtain concentrations ranging from 0.003 to 0.1 mg/kg for both the soil matrices as described in the table below:

Description	Spiking Volumes (mL)	Spiking Solutions (µg/mL)	Fortification Level (mg/kg)
Control	---	---	---
LOD	0.03	1.0	0.003
LOQ	0.1	1.0	0.01
10× LOQ	0.1	10.0	0.1

1. Twenty mL of an acetonitrile/water (99:1) solution was added to each of the samples. Samples were shaken for 30 minutes on a reciprocating shaker at approximately 180 excursions/minute and then the samples were centrifuged at 2000 ppm for 5 minutes.

2. Five-mL aliquots of each sample were transferred into a new 22 mL glass vial and 10 mL of water was added to each vial and mixed thoroughly. The final solutions were purified in a SPE experiment.

3. The clean up of ethalfluralin was performed on 300-mg multi mode (C18, SAX, SCX) SPE cartridge using the following procedure:

   a. SPE columns were placed on the manifold.

   b. The SPE column was conditioned with 3 mL of methanol followed by 5 mL of HPLC grade water.

   c. The final solution from Step 2 was transferred to the top of the SPE column. The sample was drawn through the column at a flow rate of approximately 5 mL/min, discarding the eluate. The column was dried under full vacuum (≈-10 inches Hg) for approximately 10 minutes.

   d. Ethalfluralin was eluted from the SPE column with 2 x 2.0-mL aliquots of hexane and allowing the hexane to soak into the bed of the cartridge for 1 minute before collecting the eluate in a new graduated 10 mL glass tube.

4 Three mg of peanut oil was added to each sample and the volume was adjusted to 5 mL with hexane. Samples were vortex mixed for 1 minute.

5 Samples and calibration standards were transferred to autosampler vials. They were analyzed by GC-MS with electron-impact mass spectrometry detection.

Analytical Instrumentation and Equipment

The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimisation may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

Instrumentation:	Agilent Model 6890A gas chromatograph Agilent Model 7683 autoinjector Agilent Model 5973N mass spectrometer Agilent Model G1701DA data system
Column	J & W fused silica capillary Durabond-5MS liquid phase 30 m x 0.25 mm i.d. 0.25-µm film thickness
Liner:	p/n: 5183-3316
Oven :	100 °C for 1.5 min 140 °C at 5°C/min to 150 °C at 1.0 °C/min for 3.0 min to 280 °C at 50 °C/min for 0.5 min
Transfer Line	280 °C
Carrier Gas method	Helium
Constant Flow	1.4 mL/min
Linear Velocity	~44 cm/s

Injection Method:	Splitless
Temperature:	250 °C
Pressure:	14.98 psi
Purge flow:	50.0 ml/min
Purge time:	0.90 min
Total flow:	54.2 mL/min

Electron Impact with selected ion monitoring (EI-mode)

Source Temperature	230 °C
Quad Temperature:	150 °C
Electron Multiplier	1900 volts (~200 volts above autotune)
SIM Resolution	High
Dwell Time	50 msec

Ions Monitored:	*m/z* 276 (quantitation) *m/z* 292 (confirmation 1) *m/z* 316 (confirmation 2)

Calculation of Results

For each analytical batch, a range of calibration standards was injected over the range 0.001 µg/mL to 0.1 µg/mL for ethalfluralin. A calibration curve was prepared by plotting the quantitation peak area obtained versus analyte concentration using linear regression forced through zero.

Example

Ethalfluralin recovery at 0.01 mg/kg in soil (clay loam)

ASR number = 1007/12/04

Peak area ethalfluralin= 4512

Slope of calibration curve (forced through origin) = 1159456.4083

$$\text{Ethalfluralin concentration in final extract} = \frac{4512}{1159456.4083} = 0.003891 \text{ µg/mL}$$

Sample concentration = 0.5 g/mL

Dilution factor = 1

$$\text{Ethalfluralin residue} = \frac{0.003891 \times 1}{0.5} = 0.0078 \text{ mg/kg}$$

Mean residue in control sample = 0.0000 mg/kg, (used below for background subtraction)

$$\text{Recovery} = \frac{0.0078-0.00}{0.01} \times 100 = 78\%$$

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.

Confirmation of Residue Identity

Confirmation was performed to demonstrate the selectivity of the primary method by monitoring two additional fragment ions simultaneously with the one used as the primary detection method. The analyte signal of the primary method is considered to be quantitatively correct when the retention time of the sample matches that of the standards, and the confirmation ratio for the sample is in the range of ± 20% of the average confirmation ratio found for the standards. Untreated control matrix samples and samples fortified at the lowest fortification level for each analyte/matrix combination are provided to prove selectivity of the method.

The GC-MS method is highly selective for the determination of ethalfluralin by virtue of the chromatographic separation and selective detection system used. To demonstrate further confirmation, calculations of % Recovery and % RSD were carried out on both of the confirmatory ions data (Tables 2, 3, 5 and 6).