

Final Report

**Validation of an Analytical Method for Determination of
Fluometuron and its Metabolites Des-methyl-Fluometuron and
CGA72903 in Soil**

Data Requirement

EC Guidance document on residue analytical methods,
SANCO/825/00 rev. 8.1 (16/11/2010)
OPPTS Guideline 850.6100

Test items: Fluometuron, Des-methyl-Fluometuron and CGA72903

1 Summary

A method for the determination of fluometuron, des-methyl-fluometuron and CGA72903 in soil has been successfully developed and validated. The analytical methods fulfil all requirements of guideline SANCO/825/00 rev. 8.1 (extraction efficiency has not been tested).

Extraction: Extraction with acetonitrile/water (80:20, v/v)

Cleanup: none

Final determination: HPLC-MS/MS, ESI positive ion mode (two mass transitions per test item)

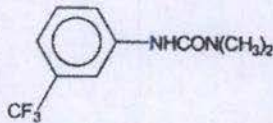
Limit of quantification: 0.01 mg/kg

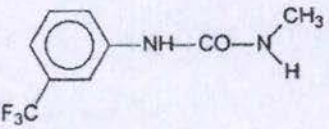
Limit of detection: below 30 % of LOQ (i.e. ≤ 0.003 mg/kg)

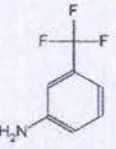
Repeatability:	The relative standard deviation (RSD) at each fortification level was below 20 % at the quantifier and qualifier mass transitions for each test item.
Blanks:	In untreated control samples the test items were not detectable (< 30 % of LOQ).
Specificity:	The method is highly specific for the test items (mass transitions from the positively charged molecule ion to two typical fragments in MS/MS mode). The retention time of the test items in solvent matched the retention times in fortified samples. No peak interferences occurred at the retention times of the test items.
Linearity:	For analysis of the test items, the detector responses were linear within the range of 0.1 ng/mL to 10 ng/mL (equivalent to 0.002 mg/kg soil to 0.20 mg/kg soil) [redacted] for the quantifier mass transitions of fluometuron, des-methyl-fluometuron and CGA72903 and for the qualifier mass transitions of des-methyl-fluometuron and CGA72903. For the qualifier mass transition of fluometuron the detector response was linear within the range of 0.1 ng/mL to 10 ng/mL (equivalent to 0.002 mg/kg soil to 0.20 mg/kg soil) [redacted]

4 Materials and Methods

4.1 Test Items

Code:	Fluometuron
Chemical name	N,N-dimethyl-N'-[3-(trifluoromethyl)phenyl]urea
CAS no.:	2164-17-2
Test item code:	2012-000468
Structure:	
Chemical formula:	C ₁₀ H ₁₁ F ₃ N ₂ O
Molecular weight:	232.2 g/mol
Purity:	99.5 %
Batch no.:	FLMT(3)-1540(V3)
Certificate of analysis:	28 February 2012
Expiry date:	28 February 2016
Supplier:	Agan Chemical Manufacturers Ltd.
Storage conditions:	Ambient temperature

Code:	Des methyl Fluometuron
Chemical name	1-Methyl-1-hydrogen-3-(α,α,α -trifluoro-m-tolyl)urea
CAS no.:	3032-40-4
Test item code:	2012-000467
Structure:	
Chemical formula:	$C_9H_9F_3N_2O$
Molecular weight:	218.2 g/mol
Purity:	99.2 %
Batch no.:	FLMT(2)-BP1-741(V2)
Certificate of analysis:	27 February 2012
Expiry date:	28 February 2016
Supplier:	Agan Chemical Manufacturers Ltd.
Storage conditions:	Ambient temperature

Code:	CGA72903
Chemical name	3-Trifluoromethyl aniline (TFMA)
CAS no.:	98-16-8
Test item code:	2011-002464
Structure:	
Chemical formula:	$C_7H_6F_3N$
Molecular weight:	161.1 g/mol
Purity:	99.5 %
Batch no.:	FLCLDN-BP14-1993 (V1)
Certificate of analysis:	09/2011
Expiry date:	09/2013
Supplier:	Agan Chemical Manufacturers Ltd.
Storage conditions:	Ambient temperature

Purity and composition

All specifications of purity and composition of the test item are provided by the sponsor/supplier.

4.2 Standard Solutions

4.2.1 Stock Solutions

A stock solution containing 1423 µg/mL of fluometuron was prepared by weighing 14.3 mg of the test item into a 10 mL volumetric flask and adjusting the volume to 10 mL with acetonitrile. This stock solution was stored refrigerated (1-10 °C) in the dark.

A stock solution containing 1061 µg/mL of des-methyl-fluometuron was prepared by weighing 10.7 mg of the test item into a 10 mL volumetric flask and adjusting the volume to 10 mL with acetonitrile. This stock solution was stored refrigerated (1-10 °C) in the dark.

A stock solution containing 1301 µg/mL of CGA72903 was prepared by weighing 65.4 mg of the test item into a 50 mL volumetric flask and adjusting the volume to 50 mL with acetonitrile. This stock solution was stored refrigerated (1-10 °C) in the dark.

4.2.2 Fortification Solutions

Combined fortification solutions (10 µg/mL and 1 µg/mL final concentration) containing all fluometuron, des-methyl-fluometuron and CGA72903 were prepared in acetonitrile/water (1:1, v/v) from the stock solutions by volumetric dilution with acetonitrile/water (1:1, v/v). All fortification solutions were stored refrigerated (nominally 1-10 °C).

4.2.3 Calibration Solutions for HPLC-MS/MS Analysis

Calibration solutions for HPLC-MS/MS analysis were prepared by volumetric dilution of the 1 µg/mL solution with acetonitrile/water (1:1, v/v) to yield intermediate dilutions of 0.1 µg/mL (100 ng/mL), 0.01 µg/mL (10 ng/mL) and 0.001 µg/mL (1 ng/mL). From these intermediate dilutions, final calibration solutions were prepared as follows:

Final concentration (ng/mL)	Standard solution 100 ng/mL (µL)	Standard solution 10 ng/mL (µL)	Standard solution 1 ng/mL (µL)	Acetonitrile/water (1:1, v/v) (µL)
10	100	–	–	900
5	50	–	–	950
2	20	–	–	980
1	–	100	–	900
0.5	–	50	–	950
0.2	–	20	–	980
0.1	–	–	100	900

4.3 Sample Material

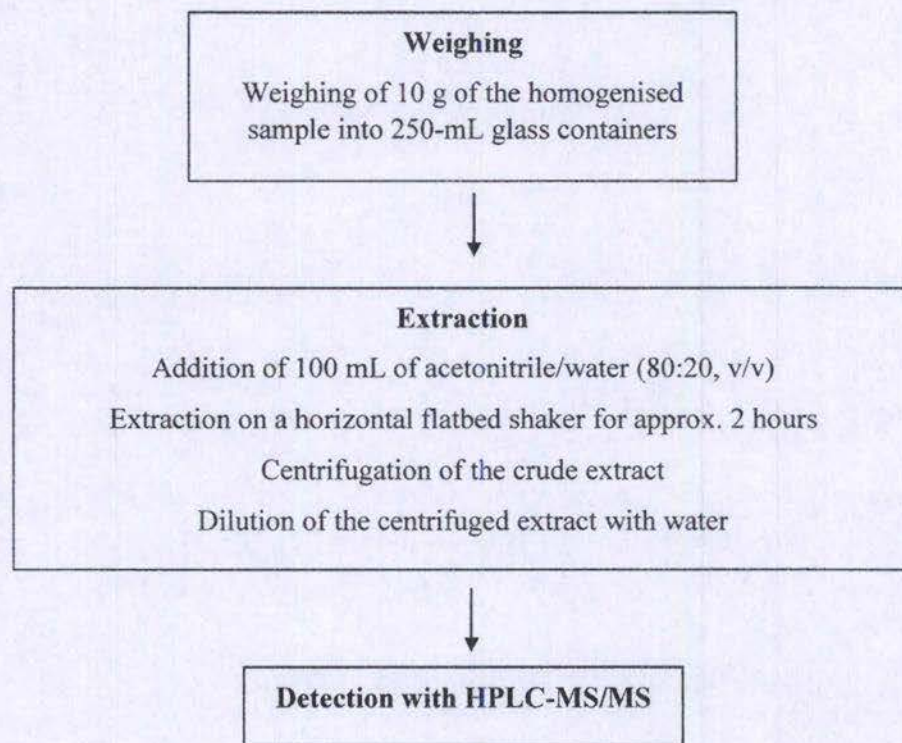
A standardised soil (loamy sand type, pH 6.6, 0.97 % organic C, 0.6 % water, soil type 2.3) obtained from LUFA Speyer / Germany was used for validation of the analytical method. The soil had been stored at ambient temperature until analysis. The data sheet is given in the appendix F in Figure 18.

4.4 Procedure for Determination of the Test Items

4.4.1 Principle of the Method

Residues of the test items were extracted from soil with acetonitrile/water (80:20, v/v). An aliquot of the extract was diluted with water after centrifugation of the crude extract and transferred into a glass vial for LC-MS/MS analysis.

4.4.2 Method Flow Chart



4.4.3 Equipment

Glass bottles (250 mL) with PTFE coated screw caps
 Centrifuge tubes with caps (single use, PE, 50 mL)
 Analytical balances (Sartorius AC 210S and TE 2101)
 Volumetric pipettes (Eppendorf): 100-1000 μ L, 10-100 μ L
 Horizontal flatbed shaker (E. Bühler)
 Centrifuge (Sigma 2-B)
 Vortex mixer (scientific Industries)
 Ultrasonic bath USR 18 (Merck)
 Autosampler glass vials for HPLC analysis
 HPLC system with MS/MS detector (Agilent HPLC system with API 5000 triple stage quadrupole mass spectrometer)

4.4.4 Reagents

For preparation of standard solutions and extraction:

Acetonitrile	Prolabo	83639.320
Water, demineralized	prepared at laboratory	

For HPLC-MS/MS analysis:

Acetonitrile	Prolabo	83639.320
Water, HPLC grade	Merck	1.15333
Formic acid	Merck	1.00264

4.4.5 Safety

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

Formic acid	Corrosive, flammable
Acetonitrile	Highly flammable, irritating

4.4.6 Extraction

The soil sample (10 g) was weighed into a 250 mL screw-cap glass bottle. The sample was extracted with 100 mL acetonitrile/water (80:20, v/v) for approximately 2 hour using a horizontal flatbed shaker.

An aliquot of the crude extract was transferred into a 50 mL centrifuge bottle and the sample was centrifuged for 5 min (7800 rpm equivalent to 6500 x g).

An aliquot (0.5 mL) of the centrifuged extract was diluted with 0.5 mL water and transferred into a glass vial for LC-MS/MS analysis.

4.4.7 Determination of Water Content

The water content of samples was determined by weighing a defined weight of the sample into an aluminium dish and determining the loss of water after drying overnight at 105 °C in a laboratory oven.

4.4.8 Analysis by HPLC-MS/MS

The extract was used for the HPLC with MS/MS detection, monitoring two characteristic mass transitions for each analyte. For fluometuron one transition (m/z 233 \rightarrow 160) was used as quantifier, the other (m/z 233 \rightarrow 72) as qualifier. For des-methyl-fluometuron one transition (m/z 219 \rightarrow 162) was used as quantifier, the other (m/z 219 \rightarrow 142) as qualifier. For CGA72903 one transition (m/z 162 \rightarrow 142) was used as quantifier, the other (m/z 162 \rightarrow 93) as qualifier. This approach follows the EC Guidance document on residue analytical method SANCO/825/00 rev. 8.1.

4.4.9 Analysis by HPLC-MS/MS

HPLC Conditions

HPLC system: Agilent LC-System with autosampler
 Column: Agilent ZORBAX Eclipse XDB-C18,
 150 x 4.6 mm, 5 µm (No. 993967-902)
 + 4 mm guard col.
 Mobile phase: A: Water + 0.5 % formic acid
 B: Acetonitrile + 0.5 % formic acid

Time (min)	% A	% B	Flow rate (mL/min)	Gradient
0.00	50	50	0.5	-
0.50	50	50	0.5	-
5.00	5	95	0.5	linear
7.00	5	95	0.5	-
7.01	50	50	0.5	-
9.00	50	50	0.5	-

Column temperature: 40 °C
 Injection volume: 50 µL
 Retention time: Fluometuron: about 5.2 min
 Des-methyl-fluometuron: about 4.7 min
 CGA72903 (TFMA): about 5.5 min

Mass Detector Conditions

Detector:	Sciex API5000
Ionization mode:	ESI
Source polarity:	positive
Curtain gas (CUR):	10 (arbitrary units)
Gas 1 (GS1):	30 (arbitrary units)
Gas 2 (GS2):	50 (arbitrary units)
Temperature (TEM):	400 °C
Interface heater (Ihe):	ON
Collision gas setting (CAD):	5
Ionspray voltage (IS):	5000
Collision cell exit potential (CXP):	18
Entrance potential (EP):	5
Declustering potential (DP):	40

Source and detection parameters for MS/MS experiments:

Compound	Parent m/z	Collision energy (V)	Fragment ions (m/z)	Ion type
Fluometuron	233.0	37	159.9	<u>Quantifier</u>
	233.0	30	71.9	Qualifier
Des-methyl-fluometuron	219.2	25	162.0	<u>Quantifier</u>
	219.2	38	142.0	Qualifier
CGA72903 (TFMA)	162.0	28	142.0	<u>Quantifier</u>
	162.0	36	92.9	Qualifier

The detector linearity was confirmed over the calibration range of interest by constructing a calibration function of peak area versus concentration within the range from 0.1 ng/mL to 10 ng/mL in acetonitrile/water (1:1, v/v).

Injections of sample extracts were interspersed with injections of quality control standards after 2-3 samples to verify the detector response and to adjust the calculated concentration as outlined below.

4.4.10 Calculation of Residues

The concentration of the test items was calculated according to the following equation by reference to the mean response of the appropriate bracketing control standards as follows:

$$R = \frac{c_1}{c_2} \cdot \frac{c_{\text{sample}}}{1000} \frac{V_{\text{Ex}}}{W} \cdot f$$

- R residue (mg/kg)
- c_1 nominal concentration of bracketed control standards (ng/mL)
- c_2 average calculated concentration of bracketed control standards, obtained from the calibration function (ng/mL)
- c_{sample} analyzed concentration of final sample, as calculated from the calibration function (ng/mL)
- f dilution factor (2)
- V_{Ex} extract volume (100 mL extraction volume + 0.06 mL water content of soil)
- W sample weight (10 g)
- 1000 conversion ng/mL → mg/L

Calculation of Water Content

The water content of the soil was determined by heating a sample aliquot (33 g) overnight at 105 °C in a laboratory oven and determining as follows:

$$c_w = \frac{W_1 - W_2}{W_1} \cdot 100$$

- c_w water content of sample (%; LUFA 2.3: 0.6 %)
- W_1 weight of moist matrix sample (g)
- W_2 weight of matrix sample after drying (g)

Recoveries were calculated by the following equation:

$$\text{Rec} = \frac{R_{\text{found}}}{R_{\text{fortified}}} \cdot 100\%$$

Rec recovery (%)
 R_{found} residue determined (mg/kg)
 R_{fortified} fortification level (mg/kg)

Exemplary calculation for a 0.1 mg/kg recovery of fluometuron (quantifier mass transition) from soil:

c₁ 5 ng/mL
 c₂ 5.40 ng/mL (mean of 5.42 ng/mL and 5.38 ng/mL)
 c_{sample} 5.06 ng/mL
 V₁ 100.06 mL
 W 10 g
 F 2

$$R = \frac{5}{5.40} \cdot \frac{5.06}{1000} \cdot \frac{100.06}{10} \cdot 2 = 0.0938 \text{ mg/kg}$$

5 Deviations from the Study Plan

The study was performed according to the study plan dated 07 March 2012 with the following deviations:

Deviation: Possible matrix effects in HPLC-MS/MS analysis were determined by comparison of the linear calibrations established from matrix matched standards and from standards in acetonitrile/water (1/1, v/v).

Reason: The determination of the matrix effect is more precisely.

Impact on Study: None

6.6 Matrix Effects

To check possible ion enhancement or suppression effects in HPLC/MS-MS analysis, final extracts from soil blank samples were spiked with defined concentrations of fluometuron, des-methyl-fluometuron and CGA72903 (10 ng/mL, 5 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.2 ng/mL and 0.1 ng/mL). A linear calibration was then established for these matrix matched standards and compared to a calibration obtained from standards in acetonitrile/water (1/1, v/v).

No significant matrix effects were observed, as detailed in appendix B. Therefore, calibration could be performed with standards in acetonitrile/water (1:1, v/v).

6.7 Specificity

The method is highly specific for analysis of the test items (mass transitions from the positively charged molecule ions to two typical fragment ions in MS/MS mode). The retention time of the test items in solvent matched the retention times in fortified samples. No peak interferences occurred at the retention times of the test items.

6.8 Storage Stability of the Test Item in Solution

Since all samples were analysed within 24 hours after extraction, the storage stability in the final extract had not to be tested.

6.9 Time Required for Analysis

For preparation of a set of 12 specimens (one validation with each 10 recoveries and 2 blanks), about 4 laboratory hours are needed. Preparation for HPLC-MS/MS including calibration standards takes an additional 0.5 hour. HPLC-MS/MS analysis can be performed unattended and requires about 10 minutes per run. Thus, a set consisting of 7 calibration standards, 10 recoveries plus 2 control standards and injections of standards after 2-3 samples to verify the detector response and retention times requires about 4 hours.

Evaluation of the HPLC-MS/MS results takes about 1 hour. Thus, the time span from initiation of extraction until completion of instrumental analysis is about 9.5 hours.