6 Guidelines

For Good Laboratory Practice:

- Chemikaliengesetz der Bundesrepublik Deutschland (ChemG) §19, sowie der Anhänge 1 und 2 in der Fassung der Bekanntmachung vom 25. Juli 1994 (BGBl. I S. 1703) und den Änderungen vom 20. Juni 2002 (S. 2090 - 2130)
- Environment Directorate OECD, Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1: The OECD Principles of Good Laboratory Practice (as revised in 1997), Env/MC/CHEM(98)17, Paris 1998

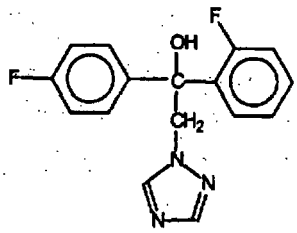
For Residue Analysis:

- European Commission, Directorate General Health and Consumer Protection, "Guidance for generating and reporting methods of analysis in support pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.", working document SANCO/3029/99 rev. 4, dated 11/07/00

7 Test and Reference Item

The test item, also used as reference item was provided by the Sponsor along with a Material Safety Data Sheet and a Certificate of Analysis. The reference item was reanalysed. Copies of the certificates are presented in Appendix 9.

Name:	flutriafol
Chemical Name (IUPAC):	(RS)-2,4´-difluoro-α-(1H-1,2,4,-triazol-1-ylmethyl)benzhydryl alcohol[1]
Chemical Name (C.A.):	(±)-α-(2-fluorophenyl)-α-(4-fluorophenyl)-1H-1,2,4-triazole-1-ethanol
Chemical Abstracts number:	76674-21-0
Chemical structure:	



Flutriafol

Batch No:	ASJ-10005-01
Purity:	99.0 %
Date of analysis:	a) 22-Jan-2004, b) reanalysis on 02-Nov-2005
Certificate No:	analytical report REF 168-01
Date of the certificate:	a) 08-Mar-2004, b) 25-Nov-2005
Expiration date:	a) Jan-2006 b) Nov-2009
Appearance:	white, solid

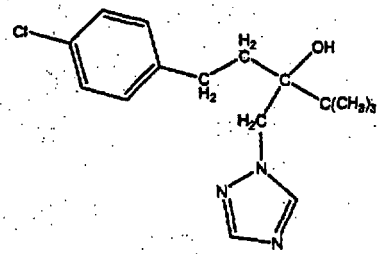
[1] The Pesticide Manual, C.D.S. Tomlin (editor), 13th edition, British Crop Protection Council, 2003

Molecular formula:	$C_{16}H_{13}F_2N_3O$
Molecular mass:	301.3 g/mol
Storage conditions:	< - 18 °C
Safety instructions:	do not take in, do not inhale and avoid skin contact

7.1 Further Active Substances

The following active substances were investigated to exclude any interference with flutriafol when applying the method of this study.

Tebuconazole:

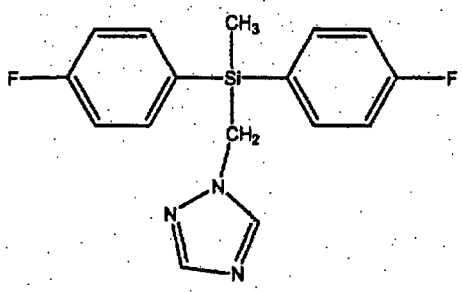
Name:	tebuconazole
Chemical Name (IUPAC):	(RS)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol[1]
Chemical Name (C.A.):	(±)-α-[2-(4-chlorophenyl)ethyl]-α-(1,1dimethylethyl)-1*H*-1,2,4-triazole-1-ethanol
Chemical Abstracts number:	107534-96-3
Chemical structure:	
Batch No:	21017
Purity:	98.0 %
Date of the certificate:	30-Oct-2002
Expiration date:	01-Oct-2008
Appearance:	colourless crystalline solid
Molecular formula:	$C_{16}H_{22}ClN_3O$
Molecular mass:	307.8 g/mol
Supplier:	Labor Dr. Ehrenstorfer-Schäfers, Augsburg, Germany
Storage conditions:	4 – 8 °C at the test facility
Safety instructions:	do not take in, do not inhale and avoid skin contact

Flusilazole:

Name:	flusilazole
Chemical Name (IUPAC):	bis(4-fluorophenyl)(methyl)(1*H*-1,2,4-triazol-1-ylmethyl)silane
Chemical Name (C.A.):	1-[[bis(4-fluorophenyl)methylsilyl]methyl]-1*H*-1,2,4-triazole
Chemical Abstracts number:	85509-19-9

[1] The Pesticide Manual, C.D.S. Tomlin (editor), 13th edition, British Crop Protection Council, 2003

Chemical structure:



Batch No:	21216
Purity:	99.5 %
Date of the certificate:	23-Jan-2003
Expiration date:	01-Jan-2007
Appearance:	colourless crystalline solid
Molecular formula:	$C_{16}H_{15}F_2N_3Si$
Molecular mass:	315.4 g/mol
Supplier:	Labor Dr. Ehrenstorfer-Schäfers, Augsburg, Germany
Storage conditions:	4 – 8 °C at the test facility
Safety instructions:	do not take in, do not inhale and avoid skin contact

8 Test System

The test system consisted of soil leaching water, groundwater and sandy soil.

9 Substrate Material

Groundwater was taken from a groundwater well in Riedstadt-Crumstad, south Hesse, Germany.

Untreated soil leaching water was collected from a test field in Riedstadt-Crumstad, south Hesse, Germany. Due to the small amount specimens of several sampling events were pooled.

Soil was collected from the same test field. The soil was characterised as a predominantly sandy loam. The material was taken from the top horizon (0 – 30 cm) and from sub-soil layers around 40 cm, 80 cm and 120 cm. The total sub-soil was combined and blended. The top soil was homogenised as well.

The soil was collected before the beginning of the study and no claim of GLP compliance is made for this sampling event and the determination of the dry weight equivalent.

10 Residue Analysis

An analytical method for determination of flutriafol in drinking, ground and surface water was supplied by the sponsor (Cheminova method 90000123, Doc No. 435-004). The extraction principle of this method was incorporated in a new method which was developed to enable a more sensitive and specific detection of flutriafol at LOQ of 0.05 µg/L in small water volumes of water. The modified method is summarised in Section 10.1 and described in detail in Appendix 1.

Starting point for developing a procedure to determine flutriafol in soil was a method supplied by the sponsor (analytical method RAM 057/04, Doc. No. 434-003). The method was modified with respect to the extraction technique, chromatography and detection. Instead of refluxing for one hour the analyte was more efficiently extracted using ASE (accelerate solvent extraction) technique. A LC-MS-MS system was used instead of a GC-NPD system in order to increase the sensitivity and specificity of the method. The modified method for soil is summarised in Section 10.2 and described in detail in Appendix 4.

10.1 Residue Analysis of Flutriafol in Groundwater and Soil Leaching Water – Summary of the Method

30 mL of soil water was extracted with dichloromethane. The extract is evaporated to dryness, reconstituted in a solvent mixture of acetonitrile/water/formic acid (50:50:0.2; v/v/v) and analysed by LC-MS-MS.

The analyte flutriafol was determined by analysing the characteristic transition m/z 302.07 → m/z 233 (cleavage of the triazole moiety). An additional transition m/z 302.07 → m/z 123 was used to confirm the results.

10.2 Residue Analysis of Flutriafol in Soil – Summary of the Method

Approximately 10 g of soil was extracted with acetonitrile/water (pH 9) by means of ASE technique. An aliquot of the extract equivalent to approximately 2 g of soil was partitioned into dichloromethane. The extract was evaporated to dryness, reconstituted in a solvent mixture of acetonitrile/water/formic acit (50:50:0.2; v/v/v) and analysed by LC-MS-MS. The analyte flutriafol was determined by analysing the characteristic transition m/z 302.07 → m/z 233 (cleavage of the triazole moiety). An additional transition m/z 302.07 → m/z 123 was used to confirm the results.

10.3 Limit of Quantification

The following limits of quantification (LOQ) of flutriafol were confirmed.

Water (groundwater and leaching water):	LOQ at 0.05 µg/L
Soil (top soil and sub-soil):	LOQ at 0.005 mg/kg

10.4 Validation

The modified methods were validated for the substrates groundwater, soil water and soil from the top layer and the corresponding sub-soil of a test field.

The groundwater and soil leaching water was fortified with flutriafol at concentration levels between nominal 0.05 µg/L and 0.5 µg/L.

Soil specimens were fortified with flutriafol at concentration levels ranging from nominal 0.005 mg/kg to 0.5 mg/kg.

As stipulated in the EC guideline on residue analytical methods (doc. Sanco/3029/99 rev.4, 11/07/00), the method was considered as valid if the following criteria were fulfilled:

- the blank values are not higher than 30 % of the LOQ
- the mean recovery at each fortification level and for each substrate is within the range of 70 -110 %, ideally with the mean within the range of 80 – 100 %
- the relative standard deviation for each fortification level and for each substrate is ≤ 20 %.

Since the proposed LC-MS-MS method was highly specific, an additional confirmatory method was not necessary.

11 Investigation for Interferences

Due to the LC-MS/MS technique the method was considered as highly specific. However, the specificity was particularly investigated with regard to the plant protection products fluslazole and tebuconacole which were applied to the test field in the past.

Standard solutions of the two agents were prepared at a concentration level of approximately 10 ng/mL using the same solvents and analysed under the same conditions as applied for the analysis of flutriafol.

12 Validation of Sampling Devices

12.1 Device for the Collection of Soil Leaching Water

The parts of the sampling devices intended to be used to collect soil leaching water were made of ceramic, plastic material, or glass. These materials were investigated by simulation the sampling procedures in the laboratory to make sure that the material did not affect the validity of the analytical results.

These tests were performed according to the following procedure:

- Purified water was fortified with flutriafol at a concentration level of about 0.2 µg/L.
- The sampling device consisting of a suction probe made of ceramic, polyamide tubes and a glass bottle were assembled in the same manner as mounted under field conditons. More technical details are illustrated in Figure A 7 - 1 of Appendix 7.
- The fortified water was passed through the device at the minimum possible vacuum. This was approximately 50 mbar. Under these conditions about 300 mL of water were drawn through the system within 3 days.
- The test was performed at ambient temperature and under reduced light.
- The concentration of flutriafol was determined according to the validated method.

The experiment was repeated once to confirm the results obtained.

12.2 Device of the Collection of Groundwater

The equipment to pump groundwater from the well into the sampling bottle (polyethylene) consisted of plastic tubes (polyvinyl chloride (PVC) and silicone rubber) and a peristaltic pump (see Figure A 7 - 2 in Appendix 7).

- Groundwater was fortified with flutriafol at a concentration level of about 0.2 µg/L
- Two units were assembled as shown in Figure A 7 - 2
- 1 L of fortified groundwater was pumped through the system into each reservoir bottle by means of the peristaltic pump.
- The test is performed at ambient room temperature.
- The concentration of flutriafol was determined according to the validated method.

14 Deviations from the Study Plan

The following items and procedures deviated from the study plan to which the section numbers refer.

Section 10: The sub-soil was taken from approximately 40 cm, 80 cm and 120 cm depth and homogenised. Therefore the homogenised specimen covers a range from approximately 40 cm to 120 cm instead of 30 cm to 90 cm.

Section 12.3: The groundwater for the storage stability test was fortified by mistake with near 0.25 µg/L of flutriafol instead of 0.20 µg/L as proposed for the nominal concentration.

Section 12.3: Groundwater was stored in glass bottles at ambient temperatures. The actual ambient temperatures ranged from 12 – 19 ° C instead of 16 – 24 °C.

Appendix 1 Residue Analytical Method of Flutriafol in Groundwater and Soil Leaching Water

A1 – 1 General Information on the Analytical Methods

The validated analytical procedures to determine flutriafol in soil and groundwater are described in this appendix. The recovery data are tabulated in Appendix 2. A typical calibration curve and typical chromatograms are provided in Appendix 3.

A1 – 2 Summary

30 mL of soil water is extracted with dichloromethane. The extract is evaporated to dryness, reconstituted in a solvent mixture of acetonitrile:water:formic acid (50:50:0.2; v/v/v) and analysed by LC-MS-MS.

The analyte flutriafol is determined by analysing the characteristic transition m/z 302.07 → m/z 233 (cleavage of the triazole moiety).

For confirmation the transition m/z 302.07 → m/z 123 is used.

A1 – 3 Equipment and Materials

A1 – 3.1 Laboratory Equipment

Shaking machine	model RSU-310R - ITS
Centrifuge	model GR 422, serial no. 29303213 - Jouan
Analytical balance	model BP 210 D, serial no. 50707526 D95-09-011 - Sartorius
Top loading balances	model 3100S, serial no. 40608510 – Sartorius model GS 3200-2, serial no. 81103161 - Kern
Ultrasonic bath	model DK 102 – Bandelin
Ultra pure water unit	model Elix 3 UV and Milli-Q 185 Plus - Millipore
Nitrogen evaporator	model NeVap 112 – Organomation model NeVap 111 – Organomation model WBR60 - ETG
Micro pipette	model Microman, different sizes - Abimed Analysen-Technik

A1 – 3.2 Glassware and Consumable Materials

Volumetric pipette*	different sizes, quality "AS" or equivalent - Hirschmann or Brand
Volumetric flask*	different sizes
Pasteur pipettes	
Graduated cylinder*	different sizes
Glass bottle*	different sizes
Sample vial	1 mL with PTFE sealed crimp-on caps - CS-Chromatographie Service
Micro vial	100 µL and 250 µL, fitting into the 1-mL sample vial
Sample vial	40 mL, 95 mm x 27 mm, glass, "EPA vial", part no. VT2400010A – AZ-Analytik
Screw cap	polypropylene (PP), part no. CT24S70105 – AZ-Analytik
Septum	SIL/PTFE, part no. CD2223025– AZ-Analytik

Note: Glassware marked with * has to be cleaned with acetone prior to use.

A1 – 3.3 Reagents

Acetonitrile	purity ≥ 99.8 %, part no. 1.14291 - Merck
Acetone	purity ≥ 99 %, part no. 1142 - Promochem
Dichloromethane	purity ≥ 99.9 %, part no. T162.1 - Roth
Formic acid	purity 98 – 100 %, part no. 1.00264, Merck
Ultra pure water	ultra pure water from the ultra pure water apparatus

A1 – 3.4 Solutions

Acetonitrile/water pH 9, (70:30, v/v)	700 mL of acetonitrile and 300 mL of water (pH 9)
Eluent A	998 mL of ultra pure water and 2 mL of formic acid
Eluent B	998 mL of acetonitrile and 2 mL of formic acid
acetonitrile/water/formic acid (50:50:0.2; v/v/v)	500 mL of acetonitrile, 500 mL of ultra pure water and 2 mL of formic acid

A1 – 3.5 Preparation of Calibration and Fortification Solutions

Stock solutions of flutriafol are prepared in acetone for calibration and fortification.

To prepare fortification solutions aliquots of the stock solution are diluted further with acetone to nominal concentrations of 100 µg/mL, 10 µg/mL and 1 µg/mL of flutriafol using volumetric pipettes and volumetric flasks.

To prepare calibration solutions aliquots of the stock solution are diluted with acetonitrile/ water/formic acid (50/50/0.2 v/v/v) to nominal concentrations ranging from 0.2 ng/mL to 10 ng/mL of the analyte.

The solutions are stored in a refrigerator at 4-8 °C.

A1 – 4 Analytical Procedure

A1 – 4.1 Extraction

Approximately 30 g of the soil water is weighed exactly into a 40-mL glass vial. Recovery specimens are fortified at this stage using a standard solution of flutriafol. 5 mL of dichloromethane is added. The capped vial is placed on a shaking machine and shaken for approximately 1 min. Thereafter the vial is centrifuged at 10 °C and 2500 rpm for 2 min. The lower organic phase is removed by means of a Pasteur pipette and transferred into another 40-mL glass vial. The solvent partition is repeated twice.

The organic phases are combined and evaporated to dryness (water bath at 40 °C). The remainder is reconstituted with 1 mL of acetonitrile/water/formic acid (50:50:0.2; v/v/v) and ultrasonically agitated for 1 minute for complete dissolution of the analyte. If necessary an aliquot of the final extract is diluted further with the same solvent mixture to fit the peak size of the analyte within the calibration range.

A1 – 4.2 LC-MS-MS Apparatus

Liquid chromatography:

HPLC apparatus	Series 1100 - Agilent, consisting of
Degasser	model G1322A
Pump	model G1311A
Injection system	model HTS Pal – CTC Analytics
Column oven	model G1316A including switching valve and splitter (1:1)
Control module	model G1323B

Detector:

LC-MS-MS System	model API 3000, serial No. D10340204 – Applied Biosystems
Vacuum-pump	model DS602 - Varian
Nitrogen generator	model NM20ZA - Peak

Data system:

Chromatography software	Analyst, Version 1.2 and 1.4.1- Applied Biosystems

A1 – 4.3 HPLC Operating Conditions

Column: C8 Hypurity, 5 µm, 150 x 3 mm – MZ Analysentechnik

Eluent A: 0.2 % formic acid in water
Eluent B: 0.2 % formic acid in acetonitrile

Gradient:

Time [min]	Eluent A (%)	Eluent B (%)
0.00	90	10
5.00	50	50
8.00	50	50
8.10	10	90
11.00	10	90
11.10	90	10
13.00	90	10

Flow: 0.6 mL/min
Injection volume: 20 µL
Column temperature: 40 °C
Sample tray temperature: 10 °C

Program for switching valve:

to waste:	0 to 4 min
to LC-MS-MS:	4 to 10 min
to waste:	10 to 13 min

Elution time: flutriafol ~ 7.3 min

A1 – 4.4 MS-MS Parameters

The parameters, in particular the gas flow conditions, are typical but may vary slightly. The exact masses of the fragments are depending on mass calibration and tuning conditions. Therefore their decimals may vary slightly.

Turbo Ion Spray:

x-axis	position 5
y-axis	position -2
Nebulizer gas	air, position 8
Curtain gas	nitrogen, position 12

Collision gas	nitrogen, position 8
Auxullary gas	air, 5500
Scanning method:	
Period	1
Retention window	0 - 10 min
Ion spray voltage	5000 V
Temperature	400 °C
Ionisation mode	ES+
Scan type	MRM
Q1 Mass	302.07 amu (parent ion)
Q3 Mass	123.00 amu (daughter ion for qualitative confirmation)
Q1 Mass	302.07 amu (parent ion)
Q3 Mass	233.00 amu (daughter ion for quantification)
Declustering potential	28 V
Focusing potential	130 V
Collision energy	39 V (m/z 302 → m/z 123 amu) 23 V (m/z 302 → m/z 233 amu)
Collision cell exit potential	8 V (m/z 302 → m/z 123 amu) 14 V (m/z 302 → m/z 233 amu)
Entrance potential	10 V
Ion energy 1	1000 V
Ion energy 3	0.500 V
Dwell	500 msec
Deflector	- 220 V
Channel electron multiplier	2200 V

A1 – 5 Calibration and Evaluation

The chromatographic system was calibrated by measuring calibration solutions interspersed between the extracts of the specimens. At least six different concentrations were measured during the analysis of one set of specimens.

The peak area of flutriafol was measured and a calibration curve was calculated using equation (1). The calibration curves ranging from 0.2 ng/mL to 10 ng/mL of flutriafol were linear with a correlation coefficient typically above 0.998.

The evaluation of the final extracts was performed by means of the regression curve.

The calibration curve and further calculations such as mean values and standard deviations were calculated according to Funk et al. The results were calculated in terms of the dimension [µg/L] assuming a density of 1.000 g/mL of the weighed water specimen.

The results were calculated using Microsoft Excel 97 SR-2 and Microsoft Office Excel 2003. The final results were rounded at the end of the calculation process. Slight deviations may be obtained and can be explained by rounding effects when recalculating the results from the data given in the report.

Examples for the calculation of the concentrations of flutriafol are demonstrated in Section A1 – 6.1.

A1 – 6 Calculation

The calibration curves were calculated from the areas of the calibration solutions versus their corresponding amount flutriafol reference item using equation (1):

(1) $$y = a + b \cdot x$$

where
- y = peak area [units]
- x = amount of flutriafol [pg]
- a = ordinate intercept [units]
- b = slope of the curve [units/pg]

The amount x of the analyte in the specimen was calculated using the transformed equation (1a):

(1a) $$x = \frac{y - a}{b}$$

where
- x = amount of flutriafol in the injected volume of the extract [pg]
- y = peak area [units]
- a, b = curve parameters from (1)

The concentration C of flutriafol in the specimen was calculated from x taking into account the injection volume, the specimen weight, and the final volume using equation (2):

(2) $$C = \frac{x \cdot V_E}{V_I \cdot W}$$

where
- C = analysed concentration of flutriafol in the specimen [µg/L]
- x = analysed amount of flutriafol in final extract of the specimen [pg]
- V_E = final volume of the extract [mL]
- V_I = injection volume [µL]
- W = specimen weight [g] = [mL]

The recovery rates were calculated according to equation (3):

(3) $$R = \frac{C \cdot 100}{C_{nom}}$$

where
- R = recovery rate [%]
- C = analysed concentration of flutriafol in the specimen [µg/L]
- C_{nom} = nominal concentration of flutriafol in the specimen [µg/L]

If blank values must be subtracted the peak size of the blank was subtracted from the fortified peak.

A1 – 6.1 Examples of Calculation

The examples for the calculation are given for the specimen 010/3248059-A deriving from the control water specimen 010/3248059 which was fortified with 0.050 µg/L of flutriafol.

30.03 g of water (specimen 010/3248059) was fortified with 1.526 ng of flutriafol, corresponding to a concentration of 0.0508 µg/L. The fortified specimen was extracted on 23-Mar-2004 and was analysed on 23-Mar-2004.

A first order curve was calculated from the data of an external calibration. The corresponding plot of this curve is shown in Figure A 3 - 1 of Appendix 3.

The content of flutriafol was calculated according to equation (1a).

(1a) $$x = \frac{13555.88 + 580.487}{463555.763} = 0.0305$$

where
- y: 13555.88, peak area after subtraction of the blank [units]
- x: amount of flutriafol [ng]
- a: -580.487, ordinate intercept [units]
- b: 463555.763, slope of the curve [units/ng]

The concentration C of flutriafol was calculated from x of equation (1 a) taking into account the injection volume, the specimen weight and the final volume using equation (2).

(2) $$C = \frac{0.0305 \cdot 1}{20 \cdot 0.03003} = 0.0508 \left[\frac{\mu g}{L}\right]$$

where
- C: concentration of the analyte [µg/L]
- x: 0.0305 ng, analysed amount of flutriafol in the injected volume
- V_E: 1.00 mL, final volume of the extract adjusted for chromatography
- V_i: 20 µL, injection volume
- W: 0.03003 kg, specimen weight [kg = L]

The recovery was calculated according to equation (3).

(3) $$REC = \frac{0.0508 \cdot 100}{0.0508} = 100 [\%]$$

where
- REC: recovery rate [%]
- C_{REC}: 0.0508 [µg/L], analysed concentration in the fortified specimen
- C_{FOR}: 0.0508 [µg/L], concentration of the fortified reference item

Appendix 4 Residue Analytical Method of Flutriafol in Soil

4 – 1 General Information on the Method

The validated analytical procedures to determine flutriafol in soil are described in this appendix. The recovery data are tabulated in Appendix 5. A typical calibration curve and typical chromatograms are provided in Appendix 6.

4 – 2 Summary of the Analytical Method

10 g of soil was extracted with acetonitrile/water (pH 9) by means of ASE technique. An aliquot of the extract equivalent to approximately 2 g of soil was partitioned into dichloromethane. The extract was evaporated to dryness, reconstituted in a solvent mixture of acetonitrile/water/formic acid (50:50:0.2; v/v/v) and analysed by LC-MS-MS. The analyte flutriafol was determined by analysing the characteristic transition m/z 302.07 → m/z 233 (cleavage of the triazole moiety).

For confirmation the transition m/z 302.07 → m/z 123 was used.

4 - 3 Equipment and Materials

4 – 3.1 Laboratory Equipment

Accelerated solvent extractor (ASE)	model ASE200 - Dionex
Centrifuge	model GR 422, serial no. 29303213 - Jouan
Shaking machine	model RSU-310R - ITS
Analytical balance	model BP 210 D, serial no. 50707526 D95-09-011 - Sartorius
Top loading balances	model 3100S, serial no. 40608510 – Sartorius model GS 3200-2, serial no. 81103161 - Kern
Ultrasonic bath	model DK 102 - Bandelin
Compressor	model 6-J - Jun-Air
Extraction cells	22 ml - Dionex
Cellulose filter	d = 19.1 mm, serial no. 3324 - Schleicher & Schuell
Ultra pure water unit	model Elix 3 UV and Milli-Q 185 Plus - Millipore
Nitrogen evaporator	model WBR60 - ETG
Drying oven	model FD 115 - WTB Binder
Bottle dispenser	model Fortuna Optifix, different sizes - Graf
Micro pipette	model Microman, different sizes - Abimed Analysen-Technik

4 – 3.2 Glassware and Consumable Materials

Evaporating dish	different sizes
Beaker	different sizes
Volumetric pipette	different sizes, quality "AS" or equivalent - Hirschmann or Brand
Volumetric flask	different sizes
Glass dispenser	different sizes
Erlenmeyer flask	different sizes
Funnel	different sizes
Pasteur pipettes	
Desiccator	
Graduated cylinder	different sizes

Glass bottle	different sizes
Sample vial	1 mL with PTFE sealed crimp-on caps - CS-Chromatographie Service
Micro vial	100 µL and 250 µL, fitting into the 1-mL sample vial
Sample vial	40 mL, 95 mm x 27 mm, glass, "EPA vial", part no. VT2400010A – AZ-Analytik
	30 mL, 140 mm x 27 mm, glass, part no. VT2400060A – AZ-Analytik
Screw cap	polypropylene (PP), part no. CT24S70105 – AZ-Analytik
Septum	SIL/PTFE, part no. CD2223025– AZ-Analytik

Note: Glassware was cleaned with acetone prior to use

4 – 3.3 Reagents

Acetonitrile	purity ≥ 99.8 %, part no. 1.14291 - Merck
Acetone	purity ≥ 99 %, part no. 1142 - Promochem
Dichloromethane	purity ≥ 99.9 %, part no. T162.1 - Roth
Ammonia	purity ≥ 25 %, part no. 1.05432 Merck
Silica gel 60	part no. 1.07734 - Merck
Sea sand	part no. 1.07712 – Merck, cleaned with acetone
Formic acid	purity 98 – 100 %, part no. 1.00264, Merck
Ultra pure water	ultra pure water from the ultra pure water apparatus

4 – 3.4 Solutions

Water	1L ultra pure water adjusted to pH 9 by the addition of ammonia
Acetonitrile/water pH 9, (70:30, v/v)	700 mL of acetonitrile and 300 mL of water (pH 9)
Eluent A	998 mL of ultra pure water and 2 mL of formic acid
Eluent B	998 mL of acetonitrile and 2 mL of formic acid
acetonitrile/water/formic acid (50:50:0.2; v/v/v)	500 mL of acetonitrile, 500 mL of ultra pure water and 2 mL of formic acid

4 - 4 Preparation of Calibration and Fortification Solutions

Stock solutions of flutriafol were prepared in acetone for calibration and fortification.

To prepare fortification solutions aliquots of the stock solution were further diluted with acetone to nominal concentrations of 100 µg/mL, 10 µg/mL and 1 µg/mL of flutriafol.

To prepare calibration solutions aliquots of the stock solution were diluted with acetonitrile/water/formic acid (50:50/0.2 v/v/v) to nominal concentrations ranging from 0.2 ng/mL to 10 ng/mL of the analyte.

The solutions were stored in a refrigerator at 4-8 °C.

4 – 5 Blending of Laboratory Specimens

The top soil specimen 010/4064641 was homogenised manually. Each of the composite sub-soil specimens 010/4080442 (used as the substrate to validate the method) and 010/4102048 (used as the substrate to investigate the storage stability) derived from 200g of each the soil specimens 010/4064653 (from around 40 cm depth), 010/4064654 (from around 80 cm depth), and 010/4064655 (from around 120 cm depth). The soil was combined and manually blended until visual homogeneity.

4 – 6 Analytical Procedure

4 – 6.1 Extraction

Approximately 10 g of the blended soil material was weighed exactly into a glass dish. Recovery specimens were fortified at this stage using a standard solution of flutriafol. After the addition of 5 g of silica gel the mixture was homogenised using a pestle.

The mixture was filled into the ASE extraction cell (22 mL) and the remaining volume was filled-up with sea sand. The extraction (ASE) was performed with a mixture of approximately 30 mL of acetonitrile:water at pH 9 (70:30; v/v) at 100 °C and a pressure of 2000 psi (number of cycles: 3; purge time: 100 sec; static time: 5 min).

4 – 6.2 Solvent Partition

Approximately 6 g of the specimen extract (equivalent to approximately 2 g soil) was filled into a 40 mL EPA vial, diluted with 20 mL of water at pH 9 and extracted 3-times using 5-mL portions of dichloromethane by manual shaking for 1 min. After each extraction step the dichloromethane phase was separated by centrifugation for 2 minutes at 2500 rpm and 10 °C.

The organic phases were combined and evaporated to dryness by a stream of nitrogen (water bath at 40 °C). The remainder was reconstituted with 4 mL of acetonitrile/water/formic acid (50:50:0.2; v/v/v) and ultrasonically agitated for 1 minute for complete dissolution of the analyte. If necessary an aliquot of the final extract was diluted further with the same solvent mixture to fit the peak size of the analyte within the calibration range.

4 – 6.3 LC-MS-MS Analysis

4 – 6.3.1 Apparatus

Liquid chromatography:

HPLC apparatus	Series 1100 - Agilent, consisting of
Degasser	model G1322A
Pump	model G1311A
Injection system	model HTS Pal – CTC Analytics
Column oven	model G1316A including switching valve and splitter (1:1)
Control module	model G1323B

Detector:

LC/MS/MS System	model API 3000, serial No. D10340204 – Applied Biosystems
Vacuum-pump	model DS602 - Varian
Nitrogen generator	model NM20ZA - Peak

Data system:

Chromatography software:	Analyst, Version 1.2 and 1.4.1- Applied Biosystems

4 – 6.3.2 HPLC Operating Conditions

Column: C8 Hypurity, 5 µm, 150 x 3 mm – MZ Analysentechnik

Eluent A: 0.2 % formic acid in water
Eluent B: 0.2 % formic acid in acetonitrile

Gradient:

Time [min]	Eluent A (%)	Eluent B (%)
0.00	90	10
5.00	50	50
8.00	50	50
8.10	10	90
11.00	10	90
11.10	90	10
13.00	90	10

Flow: 0.6 mL/min
Injection volume: 20 µL
Column temperature: 40 °C
Sample tray temperature: 10 °C

Program for switching valve: to waste: 0 to 4 min
to LC/MS/MS: 4 to 10 min
to waste: 10 to 13 min

Elution times: flutriafol ~ 7.4 min

4 – 6.3.3 MS/MS Parameters

The parameters, in particular the gas flow conditions, are typical but may vary slightly. The exact masses of the fragments are depending on mass calibration and tuning conditions. Therefore their decimals may vary slightly.

Turbo Ion Spray:

x-axis position 5
y-axis position -2
Nebulizer gas air, position 8
Curtain gas nitrogen, position 12
Collision gas nitrogen, position 8
Auxullary gas air, 5500

Scanning method:

Period 1
Retention window 0 - 10 min
Ion spray voltage 5000 V
Temperature 400 °C
Ionisation mode ES+
Scan type MRM

Q1 Mass 302.07 amu (parent ion)
Q3 Mass 123.00 amu (daughter ion for qualitative confirmation)

Q1 Mass 302.07 amu (parent ion)
Q3 Mass 233.00 amu (daughter ion for quantification)

Declustering potential	28 V
Focusing potential	130 V
Collision energy	39 V (m/z 302 → m/z 123 amu) 23 V (m/z 302 → m/z 233 amu)
Collision cell exit potential	8 V (m/z 302 → m/z 123 amu) 14 V (m/z 302 → m/z 233 amu)
Entrance potential	10 V
Ion energy 1	1000 V
Ion energy 3	0.500 V
Dwell	500 msec
Deflector	- 220 V
Channel electron multiplier	2200 V

4 – 6.4 Calibration and Evaluation

The chromatographic system was calibrated by measuring calibration solutions interspersed between the extracts of the specimens. At least six different concentrations were measured during the analysis of one set of specimens.

The peak area of flutriafol was measured and a calibration curve was calculated using equation (1). The calibration curves ranging from 0.2 ng/mL to 10 ng/mL of the analyte were linear with a correlation coefficient above 0.998.

The evaluation of the final extracts was performed by means of the regression curve.

The calibration curve and further calculations such as mean values and standard deviations were calculated according to Funk et al. /2/. The results were calculated using Microsoft Excel 97 SR-2 and Microsoft Office Excel 2003.

The final results were rounded at the end of the calculation process. Slight deviations may be obtained and can be explained by rounding effects when recalculating the results from the data given in the report.

4 – 6.4.1 Calculations

The calibration curves were calculated from the areas of the calibration solutions versus their corresponding amount flutriafol reference item using equation (1):

(1) $$y = a + b \cdot x$$

where y = peak area [cps]
x = amount of flutriafol [ng]
a = ordinate intercept [units/ng]
b = slope of the curve [units]

The amount x of the analyte in the specimen was calculated using the transformed equation (1a):

(1a) $$x = \frac{y - a}{b}$$

where x = amount of flutriafol in the injected volume of the extract [ng]
y = relative peak area [cps]
a, b = curve parameters from (1)

The concentration C of flutriafol in the specimen was calculated from x taking into account the injection volume, the specimen weight, the dry weight equivalent, and the final volume using equation (2):

(2) $$C = \frac{x \cdot V_E \cdot E_T}{V_i \cdot W \cdot E_A}$$

where C = analysed concentration of flutriafol in the specimen [mg/kg]
x = analysed amount of flutriafol in final extract of the specimen [ng]
V_E = final volume of the extract [mL]
E_T = total weight of the extract from soil material [g]
V_i = injection volume [µL]
W = specimen dry weight [g]
E_A = aliquot of the total extract taken for further clean-up and analysis [g]

The recovery rates were calculated according to equation (3):

(3) $$R = \frac{C \cdot 100}{C_{nom}}$$

where R = recovery rate [%]
C = analysed concentration of flutriafol in the specimen [mg/kg]
C_{nom} = nominal concentration of flutriafol in the specimen

If blank values had to be subtracted the peak size of the blank was subtracted from the fortified peak.

4 – 6.4.2 Examples of Calculation

The fortified soil specimen 010/4064641-A was selected as an example. The specimen derived from the untreated soil specimen 010/4064641 (top soil 0 – 30 cm). 10.02 g of the specimen was fortified with 0.05085 µg of flutriafol corresponding to a concentration of 0.00507 mg/kg.

The fortified specimen was extracted on 31-Mar-2004 and analysed on 31-Mar-2004.

The peak area of flutriafol was measured as 21479 units. Since a blank value (estimated < 0.0001 mg/kg) was detected in the corresponding non fortified specimen 010/4064641 the peak needed correction resulting in a peak area of 21334.9 units.

A linear calibration curve was used to calculate the amount of flutriafol according to equation (1a). Standard solutions with eight different concentrations were measured to generate the calibration curve. The corresponding plot of this curve is shown in Figure A 6 - 1 of Appendix 6.

The coefficients of the calibration curve were a = -1277.65 [units] and b = 528135.729 [units/ng].

Using the transformed equation (1a) the amount of flutriafol in the injected extract was

(1a) $$x = \frac{21334.9 + 1277.65}{528135.729} = 0.0428\,[ng]$$

The concentration C of was calculated from x of equation 1a and the tabulated measured data:

(2) $$C = \frac{0.0428 \cdot 4 \cdot 29.11}{20 \cdot 10.02 \cdot 6.05} = 0.0041 \left[\frac{mg}{kg}\right]$$

where C = concentration of flutriafol [mg/kg]
x = 0.0428 ng
V_E = 4 mL
E_T = 29.11 g
V_I = 20 µL
W = 10.02 g
E_A = 6.05 g

The recovery rate of the fortified specimen 010/4064641-A was calculated according to equation (3) taking into account the nominal concentration of 0.00507 mg/kg:

(3) $$R = \frac{0.0041 \cdot 100}{0.00507} = 81 [\%]$$

where R = recovery rate [%]
C = 0.0041 mg/kg
C_{nom} = 0.00507 mg/kg

The residue concentrations were calculated by means of prepared spread sheet tables.