1. Objective

This study was conducted to develop and validate an analytical method of flutolanil in soil to support the registration of the chemical as an agrochemical.

2. Testing Guidelines

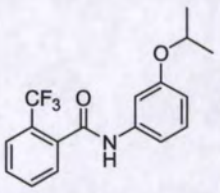
This study was designed and conducted in accordance with the following guideline:

Guidance document on residue analytical methods, SANCO/825/00 rev.7 (17 March, 2004, EU directorate general Health and Consumer Protection)

3. Materials and Methods

3.1. Test substance

Analytical standards of flutolanil was used as the test substance. Origin and identification of the test substance are shown in the table below.

Code Name	Structures	Identification	
Flutolanil		Lot No.: Purity: Expiration date: Storage requirement Inventory No.: Empirical formula: Certificate of analysis:	4AE0009C 99.5%(DOA: 2004/09/16) 2008/05/17 refrigerated in the dark 07-N-157 (received on 2007/09/18) $C_{17}H_{16}F_3NO_2$ (M.W. = 323.3) AD06088

3.1.1. Origin

Test substance used in this study was brought in Test Substance Control Unit in Research Center of Nihon Nohyaku Co., Ltd. All records for their reception, use and disposal were archived in Research Center of Nihon Nohyaku Co., Ltd.

3.2. Study design

3.2.1. Matrix

As for the matrices, Japanese soil (Kochi soil) was employed. Soil sample was obtained from Kochi station of Japan Plant Protection Association. The location of this site was described below. Details of the characterization data of soil sample are shown in Table 1. Prior to recovery tests, soil samples were

sieved through 3360 μ m sieve to remove any coarse debris might present and aliquots were subjected to moisture content determination.

Kochi Station (Japan Plant Protection Association)

1211 Fukabuchi-honda, Noichicho, Konanshi, KOCHI 781-5231, Japan

3.2.2. *Design of experiment: Recovery and Specificity*

In this study, recovery tests consisted of duplicate of control (non-fortified) and five replicates of fortified samples of two different levels of flutolanil. According to the requirement described in guidance documents in section 2, precision (intermediate or intra-laboratory precision) was not considered in this study. Final analysis was done with HPLC-MS-MS (MRM; Multiple reaction monitoring). Since MRL (Maximum residue limit) for flutolanil in/on soil was proposed as 0.05 mg/kg, the lowest and highest fortification levels were decided as 0.01 mg/kg and 0.1 mg/kg, respectively to satisfy testing guideline requirements.

Recovery experiment consisted of the following set of analysis:

Matrices	Fortification level (mg/kg)	Number of Replication	Remarks
Soil (Kochi)	Control	2	for specificity evaluation
	0.01	5	LOQ
	0.10	5	10 x LOQ

3.2.3. *Items examined and their tolerance*

1) Accuracy (trueness)

Accuracy was evaluated by relative error of determination expressed by recovery (percentage toward theoretical value), and its tolerance was 90 ± 20% (70-110%).

2) Repeatability

Repeatability, i.e. relative standard deviation within an analytical batch determined at each concentration level should not exceed 20%.

3) Specificity

Specificity (selectivity) was evaluated by analysis of blank samples. Interference derived form matrices less than 30% of lower fortification level was required.

4) Linearity

Linearity was evaluated by the correlation coefficient of calibration curve. The acceptable range was defined as more than 0.99.

3.3. Study procedure

3.3.1. Calibration

1) Preparation of calibration standard solution

Precisely weighed aliquot (ca. 10 mg) of standard of flutolanil was dissolved and filled up to 10 mL with acetonitrile. Aliquots of the obtained stock solution (ca 1000 mg/L) was mixed and further diluted with acetonitrile to give 100mg/L. The working stock solution thus obtained was then diluted with 50% acetonitrile to give the 100, 20, 10, 2, 1.0, and 0.4 μg/L. Obtained solutions were mixed up with same volume of purified blank matrix solution (5 mL/g matrix). This procedure gave standard solutions of 50, 10, 5, 1, 0.5 and 0.2 μg/L containing blank matrix of 0.1 g soil eq./mL. The calibration standard solutions thus obtained and stock solutions were stored refrigeration in the dark. Since the purity of analytical standard employed in this study was quite high (purity = 99.5%), no correction by the content was done.

2) Generation of calibration curve

Calibration standard solutions were analyzed by HPLC/MS/MS. Regression analysis between resulting peak area and amounts (concentrations) injected gave calibration curve. On the regression analysis, peak area and amounts were defined as depended variables and independent variables, respectively.

In principle, the calibration curve was renewed within every 24 hours; however, one batch of analysis was quantified with same calibration curve.

3.3.2. Specimen preparation

A schematic diagram for the following extraction and purification procedure was presented in Appendix III.

1) Fortification

To an aliquot of soil samples, necessary amount of fortification standard solution was spiked. Fortified samples thus obtained and control (blank) samples were subjected to extraction and analysis as described below.

2) Extraction

Specimens obtained were combined with 50 mL acetonitrile and vigorously shaken for 15 min and centrifuged at 2500 rpm in 5min. and then decanted supernatant. Obtained precipitate was then extracted again with 20 mL acetonitrile. Supernatant was filtrated under slight suctioning. Combined filtrates were filled up to 100 mL with acetonitrile.

3) Purification

Aliquot of extracts equivalent to 2.5 g of soil sample (12.5 mL) were withdrawn and were then combined with 3g NaCl and 12.5 mL phosphate buffer solution (0.1 M, pH 7) followed by vigorous shaking. Resultant upper layer (organic layer) was then withdrawn and evaporated *in vacuo*. Obtained residue was dissolved with acetonitrile/distilled water (4/1, v/v) 3 mL and then loaded to a carbon graphite/aminopropyl silica gel cartridge column (Envi-carb, carbon/NH_2, 500 mg/500mg, Supelco co. ltd.) conditioned with 5 mL each of acetonitrile and acetonitrile/distilled water (4/1, v/v). The cartridge column was then washed with 2 mL each of acetonitrile/distilled water (4/1, v/v) and acetonitrile. Analyte was then eluted from the column with 5 mL acetonitrile/acetic acid (95/5, v/v). The eluate was then filled up to 25 mL with distilled water.

3.3.3. HPLC/MS/MS analysis

HPLC analysis was done under the conditions described below:

1) HPLC conditions

System:	Agilent 1200 High Performance Liquid Chromatograph (Agilent technologies GmbH, Waldbronn, Germany)
Column:	Cadenza CD-C18 (ϕ 2.0 × 50 mm, 3 μm ODS, Imtakt Co., Ltd., Kyoto, Japan)
Solvent A:	Distilled water containing 0.1% formic acid (v/v)
Solvent B:	Methanol containing 0.1% formic acid (v/v)
Flow Rate:	0.20 mL/min
Column temperature:	40°C
Gradient:	Initial: 50% solvent B 0-0.5min: 50-70% solvent B in linear gradient 0.5-5.5 min: 70-100% solvent B in linear gradient 5.5-7.5 min: 100% solvent B hold

Injection Volume: 10 µL

2) Mass spectrometry

MS/MS detection was done under the conditions described below:

System: 3200Q trap triple quadrupole mass spectrometer (Applied Biosystems/MSD Sciex, Foster city, CA, USA)
Ionization: ESI (Electro spray ionization)
Polarity: Positive
Needle voltage: 5.5 kV
Curtain gas (CUR): 10 psi
Nebulizer gas pressure (GS1): 60 psi
Turbo gas temp. (TEM): 600°C
Turbo gas pressure (GS2): 80 psi
Collision gas pressure (CAD): 3 psi
Mass transient and parameters: as described below;

Analytes	Q1 [A] (amu)	Q3 [B] (amu)	Dwell Time (msec)	DP [C] (V)	EP [C] (V)	CEP [C] (V)	CE [C] (V)	CXP [C] (V)
Flutolanil	324.1	262.1	100	36	7.5	18	23	4
		242.0					35	
		282.1					19	

[A]: Mass over charge (m/z) for primary Q-pole (quasi-molecular ions)
[B]: Mass over charge (m/z) for tertiary Q-pole (product ions for detection)
[C]: These were potential for each part of Q-poles and considered as device specific.

3.3.4. Analytical quality control

Analysis by HPLC/MS/MS was done according to the following principle. 1) Prior to analyze any unknown samples; calibration standard set (6 levels) was injected. 2) Less than every 10 unknown sample analysis, QC-sample (known concentration of calibration standard) was analyzed. If the QC-samples give an acceptable peak area (100 ± 10% toward calibration standard), the analysis data for unknown samples were accepted.

3.4. Instruments, reagents and supplies

3.4.1. Instruments

Instruments used in this study are described below;

HPLC system: Agilent 1200 High Performance Liquid Chromatograph (Agilent technologies GmbH, Waldbronn, Germany)

Mass spectrometer:	3200Q trap triple quadrupole mass spectrometer (Applied Biosystems/MSD Sciex, Foster city, CA, USA) with Analyst ™ data analysis software
Electric balance:	ME235P (Sartorius GmbH, Göttingen, Germany)
Shaker:	KM Shaker V-DX (Iwaki Sangyo Co., Ltd., Tokyo, Japan)
Rotary evaporator:	N-2 (EYELA Co., Ltd., Tokyo, Japan) or equivalent
Glassware:	usual laboratory glassware such as volumetric flask, round bottom flask, and so on
Auto pipettes:	various size of Microman® (Gilson, OH, USA)

3.4.2. Reagents

Analytical reagent or HPLC grade chemicals of commercially available were used unless otherwise specified.

3.4.3. Supplies

The following solid phase extraction cartridge column was used.

Graphite carbon/NH2 cartridge:	Envi-carb® /LC-NH2 column (500 mg/500 mg, Supelco co., ltd.)

3.5. Calculations

All calculations shown below were performed by Microsoft Excel 2000 (Version: 9.0.6926 SP-3, Japanese version, Microsoft Co. Ltd.) employing maximum places allowed by the software (no rounding was done during calculation), thus manual re-calculation employing values in this report which were appropriately rounded for presentation may give slightly different results.

3.5.1. Recovery

Recovery was calculated as the percentage according to the equation shown below:

$$Recovery\ (\%) = \frac{C_d}{C_s} \times \frac{V_2}{V_1} \times 100 \qquad \text{.....(Eq. 1)}$$

Where; C_d: determination (mg/L)
C_s: spiked concentration (mg/kg)
V_1: final sample weight (kg)

V_2: final sample volume (L)

3.5.2. *Repeatability*

Repeatability (S_r) was determined as the relative standard deviation of recovery as shown in the equation below:

$$S_r = \sqrt{Vr} / M \times 100 = \sqrt{\frac{n\sum_{n=1}^{n} x^2 - \left(\sum_{n=1}^{n} x\right)^2}{n(n-1)}} / M \times 100 \qquad \text{.....(Eq. 3)}$$

Where; V_r; variance
x; individual determination
n; number of replication in one batch of analysis
M; mean value of samples data

8. Appendices

Appendix-I. Amendments, alterations and deviations of protocol

Amendment-1

Date: 2007/10/24

Detail: 1) Remove description of "and its metabolites" from 10.4.2.4 calibration range.

2) Equation for Recovery calculation will be changed to the equation shown below:

$$Recovery\ (\%) = \frac{C_d}{C_s} \times \frac{V_2}{V_1} \times 100 \qquad \text{.....(Eq. 1)}$$

Where; C_d: determination (mg/L)
C_s: spiked concentration (mg/kg)
V_1: final sample weight (kg)
V_2: final sample volume (L)

Reason: 1) Typographical error

2) Typographical error

Effects: No adverse effect on the test system and the results was expected.

Amendment-2

Date: 2007/10/24

Detail: 1) Equation for Repeatability calculation will be changed to the equation shown below:

$$S_r = \sqrt{Vr} / M \times 100 = \sqrt{\frac{n\sum_{n=1}^{n} x^2 - \left(\sum_{n=1}^{n} x\right)^2}{n(n-1)}} / M \times 100 \qquad \text{.....(Eq. 3)}$$

Where; V_r: variance
x: individual determination
n: number of replication in one batch of analysis
M: mean value of samples data

Reason: 1) Typographical error

Effects: No adverse effect on the test system and the results was expected.

Appendix-II. Unexpected circumstances during the study

None

Appendix-III. Schematic diagram of analytical method

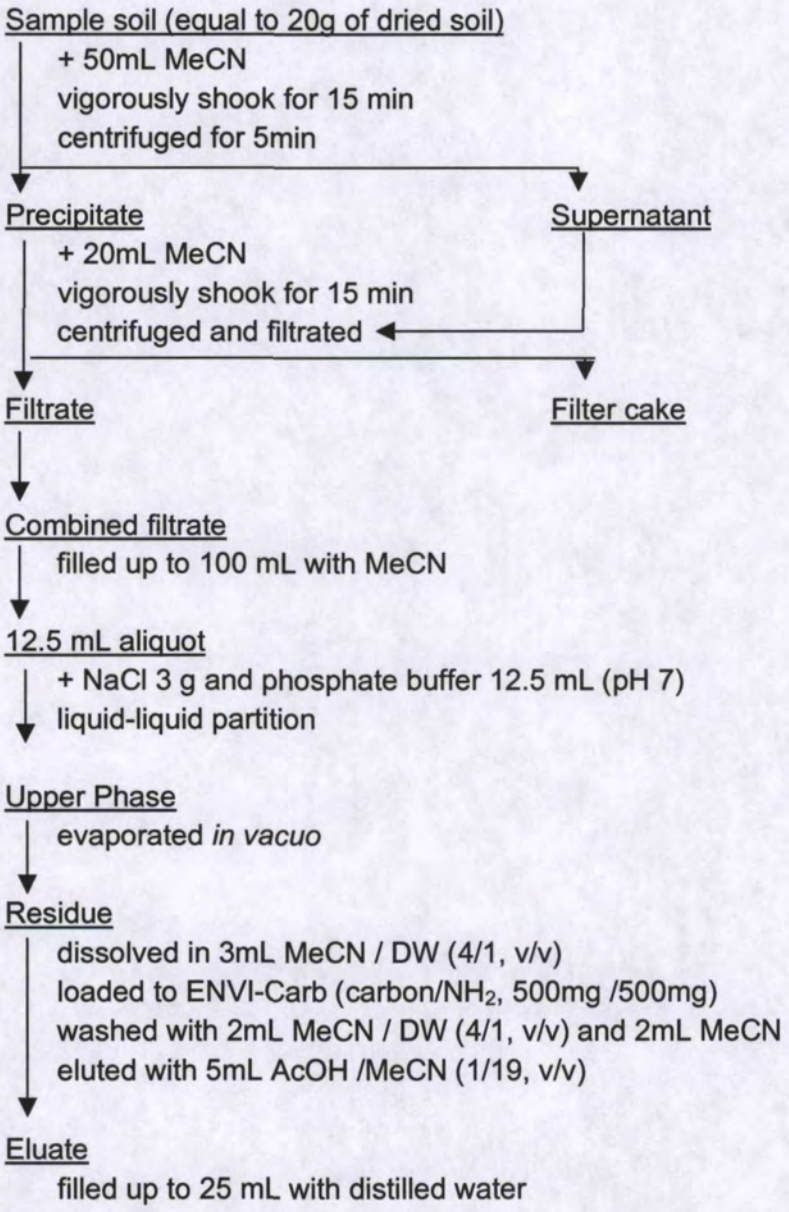


Figure III. Schematic presentation for recovery procedure