1. Objective

This study was conducted to clarify the validity of analytical method of flutolanil in water so as to determine residues of the chemical in environmental and drinking water for risk assessment purpose.

2. Testing Guidelines

This study was designed in accordance with the testing guidelines listed below.

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

2) EPA: Residue chemistry test guidelines, OPPTS 860.1340 Residue analytical method (August 1996, United States Environmental Protection Agency)

3. Materials and Methods

3.1. Test substance

9)	CAS registry number:	66332-96-5
10)	Empirical formula:	$C_{17}H_{16}F_3NO_2$ (Molecular weight: 323.3)
11)	Water solubility:	8.01 mg/L (determine by the column elution methods, 20°C) [1]
12)	Certification number:	AD08021

3.2. Study design

3.2.1. Matrix

River water was collected from Ishikawa River (Osaka prefecture) at 19th January 2011. The details for the location of water sampling were shown in Appendix III.

3.2.2. Design of experiment

In this study, recovery tests consisted of duplicated control (non-fortified) and five replicates of fortified samples at two different levels of flutolanil. Detection and quantification were done with HPLC-MS/MS (MRM; Multiple reaction monitoring). Because of high selectivity of HPLC-MS/MS method, an additional confirmatory method was not performed.

Since EU drinking water limit is 0.1 µg/L, the lowest and highest fortification levels were decided as 0.1 µg/L (LOQ; Limit of quantification) and 1.0 µg/L (10 times of LOQ), respectively to satisfy testing guideline requirements.

Recovery experiment consisted of the following set of analysis:

Matrix	Fortification level (µg/L)	Number of Replication	Remarks
Distilled water	Control	2	For specificity evaluation
	0.1	5	LOQ
	1	5	10 × LOQ
River water	Control	2	For specificity evaluation
	0.1	5	LOQ
	1	5	10 × LOQ

3.2.3. Items examined and their tolerance

To evaluate the performance characteristics, analytical method employed in this study was validated for the accuracy, repeatability and linearity, and recovery study was validated for the accuracy, repeatability and specificity.

1) Linearity

Linearity was evaluated by the correlation coefficient of calibration curve. The acceptable range was defined as more than 0.95.

2) Range

The lowest concentration that fulfills the tolerance of accuracy, repeatability and specificity (described above) was determined as the LOQ (limit of quantification). The estimated concentration that generated response 2 times grater than the noise level at the retention time of analyte on the chromatogram of the control (non-fortified) sample was determined as the LOD (limit of detection).

3) Specificity

Specificity (selectivity) was evaluated by analysis of blank samples. Interference of less than 30% of lower fortification level was required. Accuracy of recovery study was confirmed that within acceptable range.

4) Accuracy (trueness and recovery)

Accuracy was evaluated by percentage of determination toward theoretical value, and its tolerance was 100 ± 10% (90-110%) for validation test, and 90 ± 20% (70-110%) for recovery study. The highest and lowest concentration samples were evaluated.

5) Repeatability

Repeatability, i.e. square root of variance determined at each concentration level should not exceed 20%. In this study, intermediate precision or intra-laboratory precision was not considered.

6) Verification of matrix effect

The peak area of 10 µg/L standard solution with or without matrix was compared. If the area ratio was 100 ± 10% (90-110%), it was evaluated as water matrix exhibited almost no effect on the sensitivity (ionization) of flutolanil.

3.3. Study procedure

3.3.1. Determination of water quality parameters

Some parameters describing the water quality (appearance, pH), were determined as described below.

1) Appearance

Appearance of sample water was determined and recorded by naked-eye observation for its color, turbidity, visible suspending solid.

2) pH

By a well-calibrated glass-electrode pH-meter, pH of sample water was determined and recorded.

3.3.2. Preparation of calibration standard solution

A precisely weighed aliquot (9.97 mg) of flutolanil analytical standard was dissolved and filled up to 10 mL with acetonitrile. The obtained stock solution was diluted with acetonitrile to give 10 mg/L. The obtained stock solution was sequentially diluted with 50% aqueous acetonitrile (v/v) to give 500, 100, 50, 10, 5.0 and 2.0 µg/L. Standard solutions thus obtained were further diluted to give 50, 10, 5.0, 1.0, 0.50 and 0.20 µg/L. The calibration standard solutions thus obtained and stock solutions were stored below 0°C in the dark.

3.3.3. Design of stability experiment

Stability of the analyte (flutolanil) in this study was examined as calibration standard solution (dissolved in 50% acetonitrile-water). Storage condition and duration were below 0°C in the dark for 6 days.

3.3.4. Generation of calibration curve

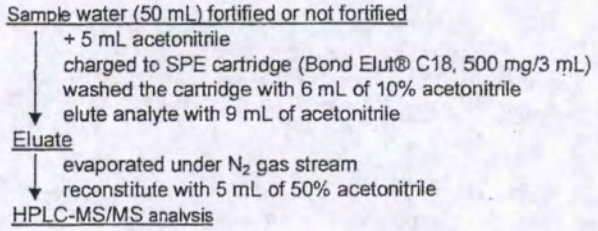
Calibration standard solutions were analyzed by HPLC-MS/MS. Regression analysis between logarithm of resulting peak area corresponding to flutolanil and logarithm of injected amounts (concentrations) gave calibration curve. On the regression analysis, peak area and injected amounts were defined as depended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

3.3.5. Preparation of samples for recovery study

To a 50 mL aliquot of blank water, 0.1 mL aliquot of 50 or 500 µg/L calibration standard solutions were added to give final flutolanil concentrations as 0.1 and 1.0 µg/L.

3.3.6. Sample pre-treatment

An aliquot (50 mL) of aqueous samples was combined with 5 mL of acetonitrile (to prevent sorption of flutolanil to glassware and keep wet the adsorbent during solid phase extraction). Samples thus obtained were loaded to solid phase extraction (SPE) cartridges that were sequentially washed with 6 mL of acetonitrile and equilibrated with 6 mL of 10% aqueous acetonitrile (v/v) prior to use. After loading of sample water, the SPE cartridges were then sequentially washed with 6 mL of 10% aqueous acetonitrile (v/v). Finally flutolanil was eluted from the SPE cartridge by 9 mL of acetonitrile. Eluate thus obtained was dried up under gentle nitrogen gas stream and reconstituted with 5 mL of 50% acetonitrile in water (v/v). A schematic diagram of the sample pre-treatment (concentrating) procedure is presented below:



3.3.7. 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 Inc.)

Column: Cadenza CD-C18 (φ 2.0 × 50 mm, 3 μm ODS, Imtakt Corp., Kyoto, Japan)

Solvent A: 0.1% formic acid in water (v/v)

Solvent B: 0.1% formic acid in methanol (v/v)

Flow Rate: 0.2 mL/min

Column temperature: 40°C

Gradient: initial: 50% solvent B
0 - 0.5 min; 50 - 70% solvent B
0.5 – 5.5 min; 70 - 100% solvent B
5.5 – 7.5 min; 100% solvent B hold
7.5 – 12 min; 50% solvent B hold
(all gradients were linear)

Injection Volume: 20 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, CA, USA)

Ionization: ESI (Electrospray ionization)

Polarity: Positive

Needle voltage: 5.5 kV

Curtain gas (CUR): 10 psi

Nebulizer gas pressure (GS1): 60 psi

Turbo gas temperature (TEM): 600°C

Turbo gas pressure (GS2): 80 psi

Collision gas pressure (CAD): 3 psi

Scan type: MRM (Multiple reaction monitoring, for quantification)

Mass transient and parameters: as described below

	Q1 [A] (amu)	Q3 [B] (amu)	Time [C] (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.

3.3.8. *Analytical quality control (QC)*

Analysis by HPLC-MS/MS was done according to the following principle. 1) Prior to analyze any unknown samples, calibration standard set 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 accuracy), the analysis data for unknown samples were accepted. If the QC-sample gave unacceptable results (out of acceptable range), the sample data were corrected by bracketing.

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 Inc.) [Binary pump; G1312A] [Column heater; G1316A] [Autosampler; G1367B] [Degasser; G1379B] [Controller; G4208A]
Mass spectrometer:	3200Q trap triple quadrupole mass spectrometer (Applied Biosystems/MSD Sciex, CA, USA) with Analyst® data analysis software (version: 4.1.1)
Electric balance:	ME235P (Sartorius GmbH, Göttingen,Germany)
Auto-pipette:	various size of Microman® (Gilson Inc, OH, USA)
Glassware:	usual laboratory glassware such as volumetric flasks, Erlenmeyer flasks, measuring cylinders (Japan industrial standard certified)
Heating block:	DTU-1B (TAITEC Co., Ltd., Saitama, Japan)
pH-meter:	D-52 (Horiba Ltd., Kyoto, Japan)

3.4.2. *Reagents*

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

3.4.3. *Supplies*

The following SPE cartridge was used. After optimization, other size SPE columns may be compatible.

SPE cartridge: Bond Elut® C18 500 mg, 3 mL (Agilent technologies Inc.)

3.5. Calculation

All calculations shown below were performed by Microsoft Excel 2002 (Version: 10.6866.6867 SP3, Japanese version, Microsoft Corp.) employing maximum places allowed by the software (no rounding was done during calculation), thus manual re-calculation employing values in this report which are appropriately rounded for presentation may give slightly different results.

3.5.1. *Accuracy*

Accuracy was calculated as the percentage of determination toward theoretical value according to the equation shown below:

$$Accuracy\ (\%) = A / B \times 100 \qquad \text{(Eq. 1)}$$

Where; C_d: determination (μg/L)

C_t: theoretical concentration (μg/L)

3.5.2. *Recovery*

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

$$Recovery\ (\%) = \frac{C_d}{C_s} \times \frac{V_2}{V_1} \times 100 \qquad \text{(Eq. 2)}$$

Where; C_d: determination (μg/L)

C_s: spiked concentration (μg/L)

V_1: initial sample volume (mL)

V_2: final sample volume (mL)

3.5.3. *Repeatability*

Repeatability (Sr) was determined as the 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