TRANSLATION

SUMMARY OF BIOCONCENTRATION STUDY REPORT

1. General items

Name of novel chemical substance (IUPAC nomenclature)	[redacted]		
Other name	[redacted]		
Structural formula or rational formula (if neither is known, outline of preparation method)	Main component [redacted] Subcomponent [redacted] Molecular formula [redacted]		
Purity of the novel chemical substance used in the study	[redacted]	Lot No. of the novel chemical substance used in the study	I-11-00
Name and concentration of impurity	[redacted]		
CAS No.	[redacted]	Vapor pressure	--
Partition coefficient	570.37 (Main component)	Molecular weight	--
Melting point	--	Property at room temperature	Brown viscous solid
Boiling point	--		
Stability	Unknown		
Solubility in various solvents	Solvent	Solubility	Stability in solvent
	Water	--	--
	DMSO	--	--
	Acetone	--	--
	Other	*1	--

[Note]
*1 Solubility in other solvents: soluble in ethanol (unknown solubility)

2. Acute toxicity test

Fish used (Latin name):	killifish *(Oryzias latipes)*	
LC50 value (96 h)	>35.0 mg/L	
Use of auxiliary	(Yes)	No
Name of auxiliary and proportion to substance studied	Crystallized sugar	20 times amount
	Megafac F-142D	20 times amount

3. Study method

Method	<Bioconcentration of Chemical Substance in Fish Body> specified in "Method for Testing New Chemical Substance, etc." (Environmental Protection Notification No. 5, Notification No. 615 of Pharmaceutical Affairs Bureau and MITI Basic Industry Bureau 1974 Notification No. 392 dated July 13, 1974 and amended on October 8, 1998) and "Bioconcentration: Flow-through Fish Test (Guideline 305, June 14, 1996)" specified in "OECD Guidelines for Testing of Chemicals."		
Fish used (Latin name):	carp (Cyprinus carpio)		
Lipid content (%)	Start: 2.94	End: 4.32	
Test substance concentration (μg/L)	First concentration level	50	
	Second concentration level	5	
Use of auxiliary		(Yes)	No
Name of auxiliary and proportion to substance studied		Name	Auxiliary concentration (μg/L)
		Crystallized sugar	First concentration level: 1000
			Second concentration level: 100
		Megafac F-142D	First concentration level: 1000
			Second concentration level: 100

4. Results

(1) Table for the result of bioconcentration test

Main component	Measurement day	After 4 days	After 7 days	After 10 days	After 17 days	After 24 days	After 28 days
First concentration level	Water tank concentration (μg/L)	46.0	45.7	51.7	53.4	53.1	51.5
	Concentration proportion		≦5.1 ≦5.1	≦5.1 ≦5.1	≦5.1 ≦5.1	≦5.1 ≦5.1	≦5.1 ≦5.1
Second concentration level	Water tank concentration (μg/L)	4.43	4.87	5.08	5.47	5.27	4.75
	Concentration proportion		≦51 ≦51	≦51 ≦51	≦51 ≦51	≦51 ≦51	≦51 ≦51

Subcomponent	Measurement day	After 4 days	After 7 days	After 10 days	After 17 days	After 24 days	After 28 days
First concentration level	Water tank concentration (μg/L)	45.7	45.3	51.7	46.5	50.7	48.4
	Concentration proportion		360 370 (370)	350 360 (350)	410 510 (460)	410 410 (410)	470 430 (450)
Second concentration level	Water tank concentration (μg/L)	4.39	5.01	5.20	5.17	5.20	4.56
	Concentration proportion		390 320 (350)	370 320 (340)	430 340 (380)	350 340 (340)	270 270 (270)

(2) Concentration proportion at steady state or upper or lower limit of concentration proportion

Main component	Concentration proportion	
First concentration level	BCFss ·(BCF)	Less than 5.1 times
Second concentration level	BCFss ·(BCF)	Less than 51 times

Subcomponent	Concentration proportion	
First concentration level	(BCFss) · BCF	440 times
Second concentration level	(BCFss) · BCF	340 times

5. Test water and fish body analytical methods

(1) Test water and fish body analysis flow

① Test water analysis

Column chromatography (SepPack C_{18}) → Analysis

② Test fish analysis

Homogenization extraction (methanol*2/formic acid (500/0.5 v/v))

→ Dilution → Analysis

*2: containing 10 mmol/L ammonium acetate

(2) Analytical instruments and conditions used

Instrument: liquid chromatography-mass spectrometer

Pump: Nippon Bunko, Model PU-980

Autosampler: Nippon Bunko, Model AS-950

Mass spectrometer: Micromass, Model Quattro II

Liquid chromatography conditions used

Column: L-column ODS (prepared by Chemical Evaluation and Research Institute), 15 cm x 2.1 mm I.D.

Column temperature: 25°C

Elution solution: Solution A (75%): methanol*2/formic acid (500/0.5 v/v)

Solution B (25%): water*2/formic acid (500/0.5 v/v)

Flow rate: 0.2 mL/min

Amount to be injected: 20 μL

Mass spectrometer condition

Ionization method: electrospray ionization (ESI)

Ion detected: cation

Detection method: selective ion monitoring (SIM)

Measurement ion: main component m/z 571

subcomponent: m/z 513

Ion source temperature: 120°C

Cone voltage: 50 V

6. Recovery rate (mean)

Recovery from test water	(%)	Main component 84.0	Subcomponent 87.1
Recovery from fish body	(%)	Main component 99.2	Subcomponent 89.9

7. Discussion

The fluctuation of the lipid content (mean) after completion of experiments in this study was 47% compared to the result before experiments, and it was over 25%. One of the causes is considered to be attributable to fluctuations in physiological conditions, feeding state, etc., causing lipid content fluctuation. Incidentally, the feeding method, etc., are to be studied further to aim for improvements in lipid content changes and fluctuations.

8. Other

Study implementation	Name:	Chemical Evaluation and Research Institute, Kurume Laboratory
	Address:	19-14 Chuo-cho, Kurume-shi, Fukuoka-ken PC830-0023
	Telephone and FAX facility	Tel: 0942-34-1500, FAX: 0942-39-6804
Study manager	Name:	Naoaki Yakata
	Years of experience:	15
Study term	November 17, 2003 - December 25, 2003	

Statement

Chemical Evaluation and Research Institute
Kurume Laboratory

Study requester: Du Pont K.K.

Study title: Bioconcentration study of [redacted] in carp

[redacted]

This final report (copy) is hereby certified to be an accurate copy of the final report of the above-titled study.

December 25, 2003
Operation manager: Hiroshi Tadokoro

Acceptance No.:	[redacted]
Test No.:	[redacted]

Final Report

Bioconcentration Study of [redacted] in Carp

December 25, 2003

Chemical Evaluation and Research Institute

Kurume Laboratory

Statement

Chemical Evaluation and Research Institute
Kurume Laboratory

Study requester: Du Pont K.K.

Study title: Bioconcentration study of [redacted] in carp

[redacted]

The above study was carried out according to the following GLP.

(1) "Standards for testing facilities specified in Ministry Ordinance Article 4 stipulating items, etc., of test related to new chemical substances and evaluation of toxicity related to designated chemical substance" (GLP) (Environmental Protection Notification 39, Notification No. 229 of Pharmaceutical Affairs Bureau and MITI Basic Industry Bureau Notification No. 85 dated March 31, 1984, amended on March 1, 2000)

(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

In addition, this final report reflects the raw data accurately, and the test data are confirmed to be valid.

December 25, 2003
Study manager: Naoaki Yakata

Reliability Guarantee Certificate

Chemical Evaluation and Research Institute
Kurume Laboratory

Study requester: Du Pont K.K.

Study title: Bioconcentration study of [redacted] in carp

[redacted]

The auditing or inspection of the above study was carried out by the reliability guarantee department of the Chemical Evaluation and Research Institute, Kurume Laboratory, and the content and date of auditing or inspection as well as dates reported to the study manager and operation manager are as follows.

Audit or inspection content	Date of audit or inspection	Date reported (study manager)	Date reported (operation manager)
Study protocol	November 17, 2003	November 17, 2003	November 17, 2003
State of implementation of study	December 25, 2003	December 25, 2003	December 25, 2003
	November 18, 2003	November 26, 2003	November 26, 2003
	November 25, 2003	November 26, 2003	November 26, 2003
	November 26, 2003	November 26, 2003	November 26, 2003
	November 27, 2003	December 2, 2003	December 2, 2003
	November 28, 2003	December 2, 2003	December 2, 2003
	December 1, 2003	December 2, 2003	December 2, 2003
	December 2, 2003	December 2, 2003	December 2, 2003
Raw data and final report	December 25, 2003	December 25, 2003	December 25, 2003

This final report is guaranteed for the test methods being accurately described, content being conformed to the study protocol and standard procedures and at the same time, raw data being reflected accurately.

December 25, 2003
Reliability Guarantee Department Manager: Shiro Horikoshi

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Tables

Figures

[redacted]

Title: Bioconcentration Test of [redacted] in Carp

Study requester: Du Pont K.K.
1-8-1 Shimomeguro, Meguro-ku, Tokyo-to PC 153-0064

Study facility: Chemical Evaluation and Research Institute, Kurume Laboratory
19-14 Chuo-cho, Kurume-shi, Fukuoka-ken PC 830-0023

Study objective: obtaining information on the extent of bioconcentration of [redacted] in carp

Study method: this study was carried out according to the following testing methods.
(1) <Bioconcentration of Chemical Substance in Fish Body> specified in "Method for Testing New Chemical Substance, etc." (Environmental Protection Notification No. 5, Notification No. 615 of Pharmaceutical Affairs Bureau and MITI Basic Industry Bureau 1974 Notification No. 392 dated July 13, 1974 and amended on October 8, 1998)
(2) "Bioconcentration: Flow-through fish test (Guideline 305, June 14, 1996)" specified in "OECD Guidelines for Testing of Chemicals."

Applicable GLP: the following standards were applied to this study.
(1) "Standards for testing facilities specified in Ministry Ordinance Article 4 stipulating items, etc., of test related to new chemical substances and evaluation of toxicity related to designated chemical substance" (GLP) (Environmental Protection Notification 39, Notification No. 229 of Pharmaceutical Affairs Bureau and MITI Basic Industry Bureau Notification No. 85 dated March 31, 1984, amended on March 1, 2000)
(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

Study schedule

Study start date	November 17, 2003
Experiment start date	November 21, 2003
Experiment completion date	December 19, 2003
Study completion date	December 25, 2003

Storage of study documents and materials

(1) Study materials

The samples provided by the test requester are to be stored, after sealing in storage containers for 10 years from the notification corresponding to the Article 4, Clause 1, No. 1, 2 or 3 of the law related to regulations for evaluation, production, etc., of chemical substances (abbreviated to "Chemical evaluation law," below), in the sample storage room of the Kurume Laboratory. After the prescribed storage term, the actions on the samples are to be determined after consultation with the test requester. However, if the product quality of a sample is expected to change markedly during the storage term, the term is set to be the one for the product quality withstanding storage, and at the time of disposal, an approval is to be obtained from the study requester.

(2) Raw data, documents, etc.

The raw data, study protocol, study request, study substance survey form and other necessary documents together with the final report are to be stored for 10 years from the notification corresponding to the Article 4, Clause 1, No. 1, 2 or 3 of the Chemical Evaluation Law in the document storage room of Kurume Laboratory. The actions on these materials after the storage term are to be determined after consultation with the test requester.

Study personnel

Study manager	Naoaki Yakata Affiliation: Second Testing Department
Study investigators (Implementation of bioconcentration test)	Maki Saeki Yoshiyuki Inoue Shin-ichiro Kaku Yurika Mori Akiko Hiroshige Akemi Inoue Yasuro Kawashima Chirise Fukuda
Rearing control manager	Yasuro Kawashima
Acute toxicity test investigators	Yasuro Kawashima Tadayoshi Fujiuchi

Approval of final report December 25, 2003

Study manager: Naoaki Yakata

Summary

Study title

Bioconcentration test of [redacted] in carp

Study conditions

Acute toxicity test

(1) Fish used	Killifish
(2) Exposure time	96 h
(3) Exposure method	Semistatic type (water exchange every 8-16 h)

Bioconcentration test

(1) Fish used	Carp
(2) Concentration tested	First level: 50 μg/L
	Second level: 5 μg/L
(3) Exposure time	28 days
(4) Exposure method	Continuous water flow system
(5) Analytical method	Liquid chromatography-mass spectrometer combination

Results

(1) 96 h LC50	>35.0 mg/L
(2) Bioconcentration factor at steady state	
Main component	First level: -
	Second level: -
Subcomponent	First level: 440 times
	Second level: 340 times
(3) Bioconcentration factor	
Main component	First level: less than 5.1 times
-	Second level: less than 51 times
Subcomponent	First level: 350-510 times
	Second level: 270-430 times

1. Test substance

[redacted]n this report has the following name and other related items.

1.1 Name*1

[redacted]

1.2 Structural formulas and others

Structural formulas

[redacted]

[redacted]

Molecular formula [redacted]

Molecular weight [redacted]

CAS No. [redacted]

*1 From documents provided by the study requester

2. Sample provided by study requester

In the following the sample provided by the study requester is called the sample provided.

2.1 Provider and Lot No.*1

(1) Provider: Du Pont K.K.

[redacted]

2.2 Purity*1

[redacted]

(2) Impurity [redacted]

2.3 Confirmation of study substance

The infrared absorption spectrum provided by the study requester was confirmed to coincide with the spectrum measured at the Kurume Laboratory (see Figure 13, Reference 2).

2.4 Physicochemical properties*1

State at room temperature	Brown viscous solid
Solubility	Soluble in ethanol (unknown solubility)
Stability	Unknown

*1 Information in documents provided by the study requester

2.5 Storage conditions and stability

(1) Storage condition Storage at a room temperature in a dark place

(2) Stability confirmation The infrared absorption spectrum measurement was carried out before and after experiments, and as a result, the two spectra coincided confirming the substance was stable under the storage conditions used (see Figure 13).

2.6 Stability under test conditions

A preliminary study was carried out before starting experiments to confirm the substance was stable under the study conditions used.

3. Acute toxicity test

3.1 Testing method

The method used was the method of "Industrial wastewater testing method – acute toxicity test in fish" (71 of JIS K 0102-1998).

3.2 Fish used

(1) Kind of fish Killifish Oryzias latipes

Reason for selection: sensitivity similar to that of carp and readily available

(2) Source Nakajima Fish Farm
(2029 Ohaza Nagasu, Nagasu-cho, Tamana-gun, Kumamoto-ken 869-0123)

(3) Rearing and storing conditions

Term: upon delivery, those observed to be abnormal by naked eye observation were removed, the remaining fish were exposed to a medicinal bath for prevention of diseases and extermination of parasites and reared for 44 days in a state of flowing water.

Medicinal bath: For disease prevention, a mixture of 20 mg/L of Elverju [transliteration] and 7 g/L sodium chloride was used as a medicinal bath for 24 h. For parasite extermination, a bath of 30 μL/L of formalin for 24 h was used twice.

(4) Conditioning conditions

Term: after rearing and storing, the fish were transferred to a conditioning tank for medicinal bathing. Subsequently, they were conditioned in a state of flowing water at 25 ± 2°C for 7 days. If any abnormality was observed during that period, the individual was removed.

After reselection and medicinal bathing, the selected fish were conditioned in a state of flowing water for 18 days.

Medicinal bath: After transferring to a conditioning tank, a mixture of 20 mg/L Elverju and 7 g/L sodium chloride was used to carry out medicinal bathing for 24 h.

After reselection, a mixture of 20 mg/L Elverju and 7 g/L sodium chloride was used to carry out medicinal bathing for 24 h.

(5) Body weight Mean of 0.17 g
(6) Body total length Mean of 2.8 cm
(7) Sensitivity test

LC50 with a standard substance PCP-Na (pentachlorophenol sodium, reagent, manufactured by Tokyo Kasei Kogyo) for 48 h was 0.755 mg/L in the case of test fish of the same lot.(TFO-031020).

3.3 Water used for the test
(1) Kind of water

Underground water pumped up from the ground of Kurume Laboratory.

(2) Water quality confirmation

The results of water quality analyses carried out by sampling on November 11, 2003 at the Kurume Laboratory are shown in Reference 1 (measurement frequency: once/6 months).

The items used in the analyses of the water used for the test were confirmed to conform to one of the following standards.

① "Water quality standard based on the Water Works Law" (amended December 21, 1992, Ministry of Health and Welfare Ordinance No. 69)
② "OECD Guidelines for Testing of Chemicals," "Fish, Early-life Stage Toxicity Test (Guideline 210, July 17, 1992)"
③ "Standard for Fishery Water" (Japanese Association of Aquatic Resources Protection, March 1983)
④ "Environmental standards related to water pollution" (amended on February 22, 1999, Environment Protection Agency Notification No. 14)
⑤ "OECD Guidelines for Testing of Chemicals," "Bioconcentration: Flow-through Fish Test (Guideline 305, June 14, 1996)"

3.4 Test conditions

(1) Test tank	Round-glass water tank	
(2) Amount of test solution	5 L/concentration level	
(3) Test temperature	Start of exposure	24.8°C
	Before water exchange	25.1°C
(4) Dissolved oxygen concentration	Start of exposure	8.1 mg/L
	Before water exchange	7.8 mg/L
(5) pH	Start of exposure	8.0
	Before water exchange	7.8
(6) No. of fish tested	10/concentration level	
(7) Exposure time	96 h	
(8) Exposure method	Semistatic type (water exchange in every 8-16 h)	

3.5 Stock solution preparation method

(1) Dispersant

Crystallized sugar
Megafac F-142D

(2) Preparation method

The sample provided and a 20 times amount of crystallized sugar were stone-milled; subsequently, Megafac F-142D in a 20 times amount of the amount of the sample provided was

added, and the mixture was stone-milled further. Subsequently, ion-exchanged water was added to prepare a stock solution with a test substance concentration of 500 mg/L.

3.6 Test implementation

(1) Test site Room 214LC50

(2) Test date November 17, 2003 – November 21, 2003

3.7 Calculation of 96 h LC50

The Doudoroff method was used.

3.8 Test result

96 h LC50 of test substance >35.0 mg/L*2 (see Figure 3)

*2: The concentration of the dispersant (Megafac F-142D) used in the above case was 700 mg/L, the 96 h LC50 of the dispersant was 7140 mg/L, and considering the effect of the toxicity of the dispersant, no test above this concentration was carried out.

4. Implementation of bioconcentration test

4.1 Fish used

(1) Kind of fish Carp Cyprinus carpio

Reason for selection: for considering consistency with past findings and size being easy to handle

(2) Source Sugishima Fish Farm
(123-2 Gunchikuichiban-cho, Yatushiro-shi, Kumamoto-ken 866-0024)
Date of receiving tested fish August 14, 2003

(3) Rearing and storing conditions

Term: upon delivery, those observed to be abnormal by naked eye observation were removed, the remaining fish were exposed to a medicinal bath for prevention of disease and extermination of parasites and reared for 18 days in a state of flowing water.

Medicinal bath: for disease prevention, a mixture of 50 mg/L of fishery OTC (oxytetracycline hydrochloride) and 7 g/L sodium chloride was used as a medicinal bath for 24 h. For parasite extermination, a bath of 30 μL/L formalin for 24 h was used twice.

(4) Conditioning conditions

Term: after rearing and storing, the fish were transferred to a conditioning tank for medicinal bathing. Subsequently, they were conditioned in a state of flowing water at 25 ± 2°C for 36 days. If any abnormality was observed during that period, the individual was removed.

After reselection, transfer to a test tank, and medicinal bathing, the selected fish were conditioned at the same temperature as above in a state of flowing water for 42 days.

Medicinal bath: after transferring to a conditioning tank, a mixture of 50 mg/L of fishery OTC and 7 g/L sodium chloride was used to carry out medicinal bathing for 24 h.

In the test tank, a mixture of 20 mg/L of Elverju and 7 g/L sodium chloride was used to carry out medicinal bathing for 24 h.

(5) Body total length	7.0-9.0 cm
(6) Lot	TFC-030814
(7) Age	Less than one year old
(8) Feed	
Kind	Compounded feed for young carp
Composition	Protein content of 43.0% or higher
	Fat content of 3.0% or higher
Manufacturer	Nippon Haigo Shiryo K.K.
Feeding method	Amount corresponding to about 2% of the body weight of test fish was fed in two portions a day (once a day on holidays).
	No feeding for 24 h prior to sampling of study fish.

4.2 Water used for the test

Same as in 3.3.

4.3 Test and environment conditions

(1) Test water feeding method	The feeding was carried out by using a flowing water device assembled at the Kurume Laboratory	
(2) Test tank	100-L volume glass water tank	
(3) Amount of test water	With a proportion of 2 mL/min of the stock solution and 800 mL/min of test water, 1155 L/day were fed to the test tank.	
(4) Stock solution tank	25 L glass bottle	
	Replacement frequency	Once/week
(5) Test temperature	First concentration level	25.0-25.2°C

	Second concentration level	25.0-25.2°C
	Control group	25.0-25.1°C
(6) Dissolved oxygen concentration	First concentration level	7.6-8.1 mg/L
	Second concentration level	7.8-8.1 mg/L
	Control group	8.1 mg/L
(7) pH	First concentration level	7.7-8.0
	Second concentration level	7.7-8.0
	Control group	7.6-8.0
(8) Illumination time	Artificial illumination by white fluorescent lamps (14 h light/10 h dark)	
(9) No. of fish tested	First and 2nd concentration levels: 38 (start of exposure)	
	Control group: 12 (start of exposure)	
(10) Exposure time	28 days	
	Reason: reached steady state after 28 days	
(11) Experiment site	213 Aquatron Room	

4.4 Stock solution preparation method

(1) Dispersant

Same as that in section 3.5 (1).

(2) Preparation method

• First concentration level

A stock solution having a test substance concentration of 20 mg/L was prepared by using the same procedures as those in section 3.5 (2).

• Second concentration level

A stock solution having a test substance concentration of 2 mg/L was prepared by using the same procedures as those in section 3.5 (2).

• Control group

Crystallized sugar and Megafac F-142D were dissolved in ion-exchanged water to prepare a stock solution having concentrations of 400 mg/L.

4.5 Test concentrations

Considering the 96 h LC50 result and test substance analytical sensitivity, the test substance concentrations were set as follows.

First concentration level 50 μg/L
Second concentration level 5 μg/L
At the same time, a control group was set as a blank test.

4.6 Observation, measurement and cleaning

(1) Observation of test fish: state of health, etc., observed twice a day by the naked eye.
(2) Amount of test water: measured once a day with a graduated cylinder and the result recorded.
(3) Test temperature: measured once a day with an alcohol thermometer and the result recorded.
(4) Dissolved oxygen concentration: measured twice a week with a dissolved oxygen meter and the result recorded.
(5) pH measurement: measured once or more a week with a pH meter and the results recorded.
(6) Cleaning: during the experiment term, the carp excreta, dirt on the water tank, etc., were cleaned once a day.

4.7 Test water and test fish analyses

The analyses of the test substance in the test water and fish were carried out by the liquid chromatography-mass spectroscopy (LC-MS) method.

When the test substance was analyzed by liquid chromatography-mass spectrometry, two peaks were detected. Therefore, quantitative determination was carried out for each peak. However, the concentration of each peak shown does not take into account the component composition as a test substance concentration shown in the standard solution preparation in section 4.7.3 (2).

4.7.1 Frequency of analysis

(1) Test water

For both the first and second concentration levels during exposure, the test water analysis was carried out once before and then throughout the first test fish analysis. One sample per analysis was used.

(2) Test fish

For both the first and second concentration levels during exposure, the test fish analysis was carried out 5 times, the number of fish used per analysis was 4, and they were divided into 2 groups (2 per group)[*3].

In the control group, analysis was carried out before and after the experiment, 4 per analysis were used, and they were divided into 2 groups (2 per group). In addition, for fat measurement, 2 were selected making the total of 3 groups (2 per group).

*3: because the amount of sample reserved for fat content measurement from only 1 fish was insufficient, 2 per group were used.

4.7.2 Pretreatment of analytical sample

(1) Test substance in test water

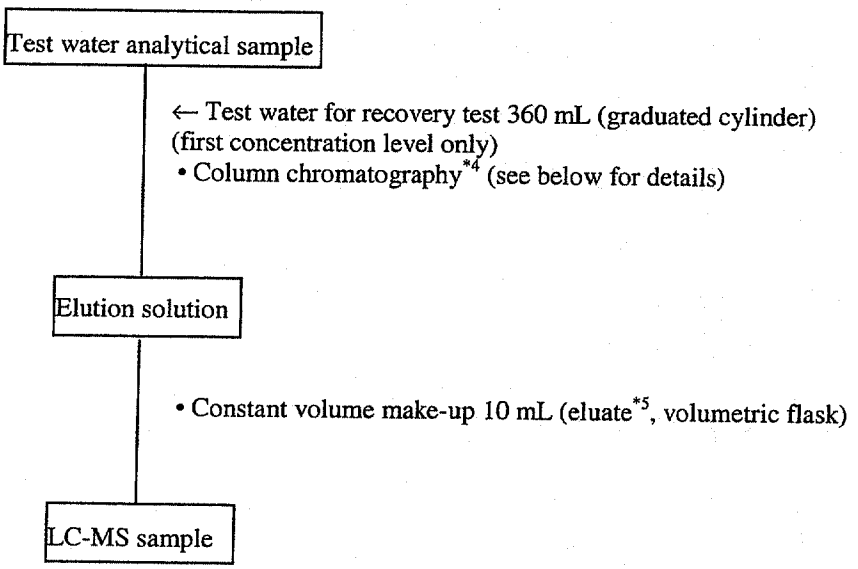
From the test tanks,

First concentration level: 40 mL

Second concentration level: 400 mL

were collected, and a pretreatment was carried out according to the following scheme to obtain liquid chromatography-mass spectroscopy (LC-MS) samples.

Scheme



*4 Column chromatography conditions

Sep-Pak C_{18}

(Washing method: eluate*5, water*6 10 mL each)

Loading method: whole amount loaded

Elution method: first solution water*6 5 mL

second solution eluate*5 8 mL

Second solution used for analysis

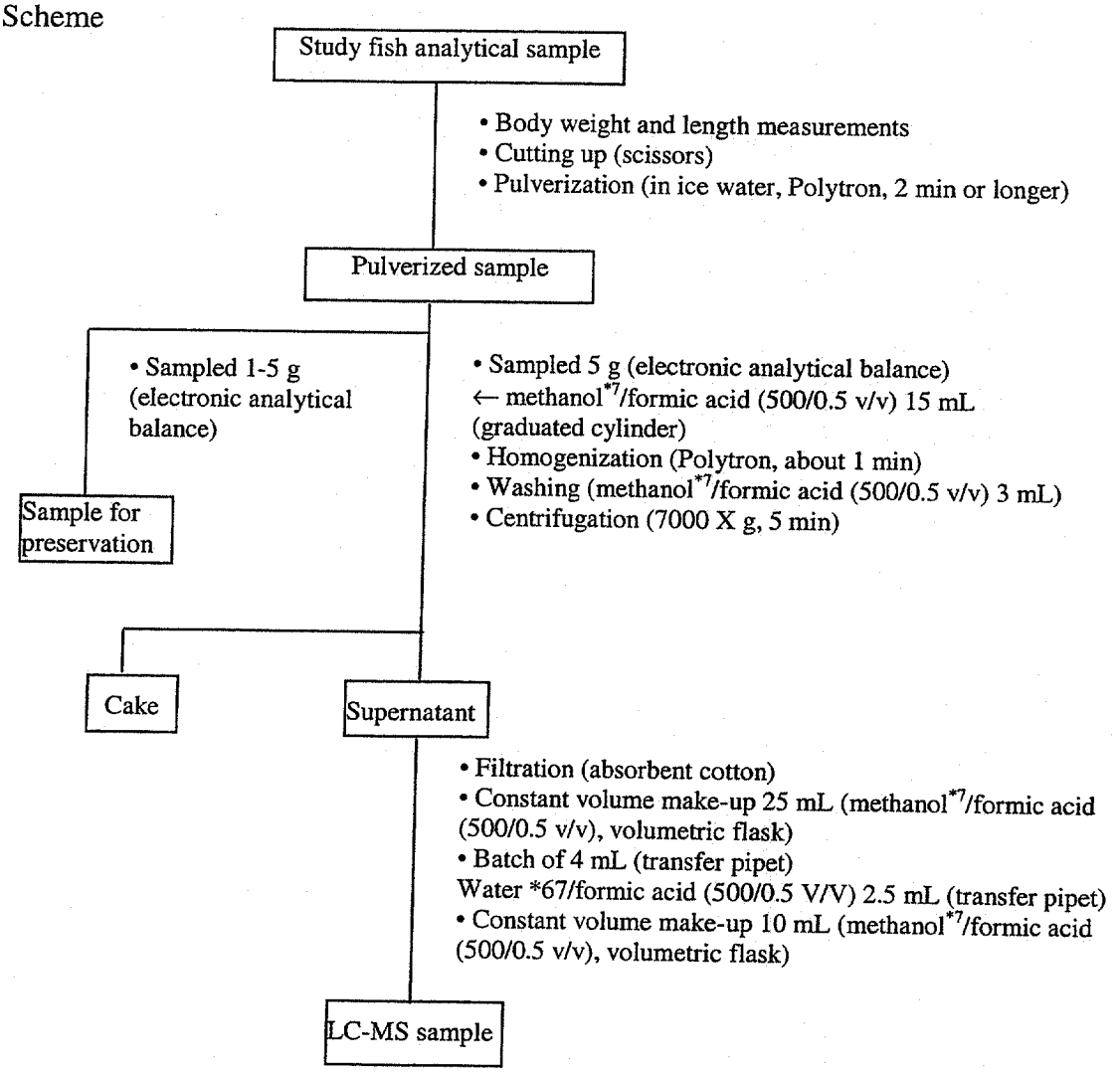
*5 See quantitative determination conditions in section 4.7.3 (1)

*6 Purified water prepared from tap water using a super-pure water system

(2) Study substance from study fish

The study fish were taken from the test tank, and a pretreatment was carried out according to the following scheme to obtain a liquid chromatography-mass spectrometry (LC-MS) sample.

Scheme



*7: containing 10 mmol/L ammonium acetate

4.7.3 Quantitative analysis of test substance

LC-MS samples prepared by the above pretreatment were used in the test substance analysis by combined liquid chromatography-mass spectroscopy based on the following quantitative determination conditions. Incidentally, in the case of test fish analyses, if the test substance concentration was outside the range of the calibration curve, the sample was diluted

before analysis to bring the concentration within range. The test substance concentration in an LC-MS sample was determined by comparing peak areas of mass fragmentograms of standard solutions and LC-MS sample and extrapolating (see Tables 5 and 6, Figure 6, Tables 8, 9 and 10 and Figures 9, 10 and 11).

(1) Quantitative determination conditions

Instrument:	combined liquid chromatographer-mass spectrometer
Pump:	Nippon Bunko Model PU-980
Autosampler:	Nippon Bunko Model AS-950
Mass spectrometer:	Micromass Model Quattro II

Liquid chromatography conditions

Column: L-column ODS (prepared by Chemical Evaluation and Research Institute)
15 cm x 2.1 mm I.D.

Column temperature: 25°C

Elution solution: Solution A (75%): methanol[*7]/formic acid (500/0.5 v/v)
Solution B (25%): water[*6,7]/formic acid (500/0.5 v/v)

Flow rate: 0.2 mL/min

Amount injected: 20 μL

Mass spectrometer conditions

Ionization method:	electrospray (ESI)	
Ion detected:	cation	
Detection method:	selective ion monitoring (SIM)	
Ion measured:	Main component	m/z 571
	Subcomponent	m/z 513
Ion source temperature:		120°C
Cone voltage:		50 V

(2) Standard solution preparation

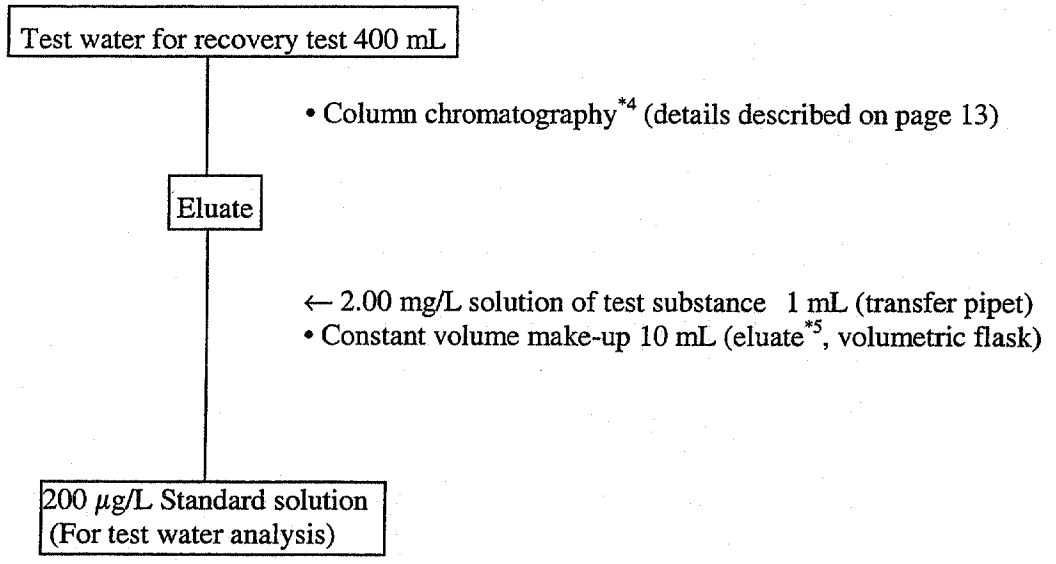
The standard solution for determining the test substance concentration of an analytical sample was prepared as follows.

(a) Test water analysis

100 mg of the provided sample weighed accurately were dissolved in methanol to obtain a 1000 mg/L solution of the test substance. It was diluted with the elution solution[*5] to prepare a

2.00 mg/L solution of the test substance, which was used to carry out a pretreatment according to the following scheme to obtain a 200 μg/L standard solution.

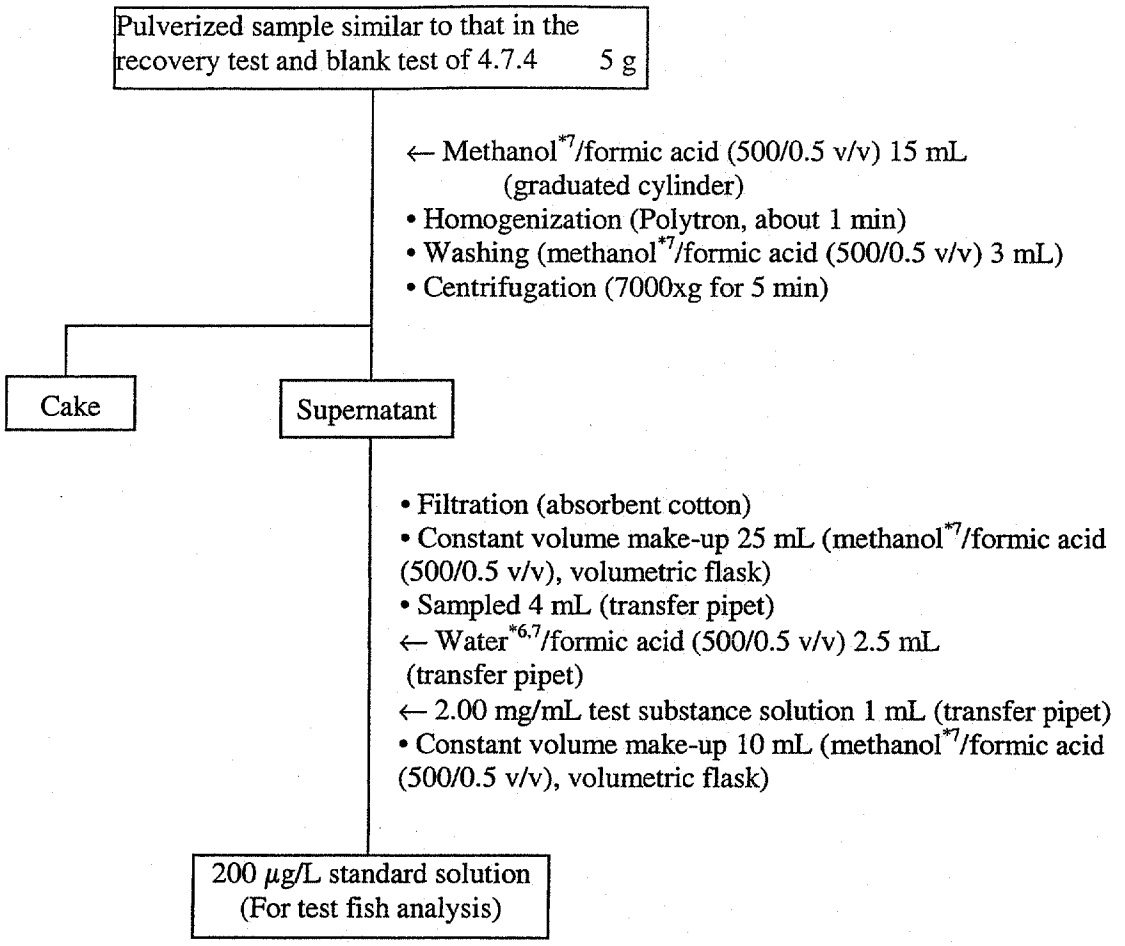
Scheme



(b) Test fish analysis

100 mg of the provided sample weighed accurately were dissolved in methanol to obtain a 1000 mg/L solution of the test substance. It was diluted with the elution solution*5 to prepare a 2.00 mg/L solution of the test substance, which was used to carry out a pretreatment according to the following scheme to obtain a 200 μg/L standard solution.

Scheme



(3) Calibration curve preparation

(a) Test water analysis

The same procedures as those used for the preparation of the standard solution in (2) (a) were carried out to prepare 100, 200 and 400 μg/L standard solutions. These standard solutions were analyzed under the quantitative determination conditions of (1), and a calibration curve was prepared from the peak areas on respective mass fragmentograms and concentrations.

The quantitative determination lower limits of the peak areas were set as

Main component: 7000 (test substance concentration of 25 μg/L)

Subcomponent 1000 (test substance concentration of 12 μg/L)

by considering the noise level (see Figure 4).

(b) Test fish analysis

The same procedures as those used for the preparation of the standard solution in (2) (b) were carried out to prepare 100, 200 and 400 μg/L standard solutions. These standard solutions

were analyzed under the quantitative determination conditions of (1), and a calibration curve was prepared from the peak areas on respective mass fragmentograms and concentrations.

The quantitative determination lower limits of the peak areas were set as

Main component: 7000 (test substance concentration of 20 μg/L)
Subcomponent 1000 (test substance concentration of 19 μg/L)

by considering the noise level (see Figure 7).

4.7.4 Recovery test and blank test

(1) Method

To determine the recovery rate of the test substance in the analytical procedures for the test water and test fish described in section 4.7.2, the test substance stock solution was added to the recovery test water and thinly sliced fish (10 g), and recovery tests were carried out. Furthermore, blank tests were carried out for the recovery test water and thinly sliced fish with no test substance added by carrying out the same procedures as those used for the above recovery test. For the recovery and blank tests, the measurements were carried out for 2 items each.

(2) Results

As a result of measurement carried out by the method of (1), no peaks were observed at the positions of the test substance in the mass fragmentograms obtained in the blank tests. The recovery rates and mean recovery rate for 2 items each in the analytical procedures were as follows, and the mean recovery rate was used as a correction in the case of determining the concentration of the test substance in an analytical sample (see Tables 4 and 7 and Figures 5 and 8).

Recovery rate in the analytical procedures

Test water analysis (2000 ng of test substance added)

Main component	82.7%, 85.3%	Mean 84.0%
Subcomponent	84.7%, 89.6%	Mean 87.1%

Test fish analysis (25000 ng of test substance added)

Main component	99.5%, 98.9%	Mean 99.2%
Subcomponent	90.3% 89.5%	Mean 89.9%

4.7.5 Fat content of test fish

A pulverized test fish sample of the control group was extracted with chloroform/methanol, and the fat content was determined by weight analysis.

4.7.6 Calculation of test substance concentration in analytical sample and quantitative lower limit

(1) Calculation of test substance concentration in test water analysis sample

The calculation was carried out according to the formulas shown in Tables 5 and 6; the results shown were rounded to 3 significant figures.

(2) Quantitative lower limit concentration of test substance in test water

From the quantitative lower limit determined in the preparation of the calibration curve in section 4.7.3 (3) (a), the qualitative lower limit concentrations*8 were calculated as follows.

Main component	First concentration level	7.5 μg/L
	Second concentration level	0.75 μg/L
Subcomponent	First concentration level	3.5 μg/L
	Second concentration level	0.35 μg/L

(3) Calculation of test substance in test fish analytical sample

The calculation was carried out according to the calculation formulas shown in Tables 8, 9 and 10; the results shown were rounded to 3 significant figures.

(4) Quantitative lower limit concentration of test substance in test fish

From the quantitative lower limit determined in the preparation of the calibration curve in section 4.7.3 (3) (b), the qualitative lower limit concentrations*8 were calculated as follows when the amount of pulverized test fish sample was 5 g.

Main component	260 ng/g
Subcomponent	270 ng/g

*8: Quantitative lower limit concentration of test substance (μg/L or ng/g)

$$= \frac{A}{B/100 \times CxE/D}$$

A : calibration curve quantitative lower limit concentration (μg/L)

B : recovery rate (%)

C : amount of test water sampled (mL) pulverized test fish sampled (g)

D : final amount of liquid (mL)

E : Sampling ratio

The calculation results were rounded to 2 significant figures.

4.7.7 Calculation method for total mean test substance concentration in test water during exposure term

$$\overline{Cwt} = \{Cw(1) + \cdots\cdots + Cw(n)\} / n$$

$\overline{Cwt}$: total mean test substance concentration in test water (μg/L)

n: No. of test water analyses (frequency of measurement)

Cw(1): test substance concentration in test water measured for the 1st time (μg/L)

Cw(n): test substance concentration in test water measured for the nth time (μg/L)

4.7.8 Calculation method for bioconcentration factor (BCF)

The bioconcentration factor (BCF) was calculated according to the following formulas.

(1) Calculation of mean test substance concentration in test water for calculation of bioconcentration factor

$\overline{Cw}$ = [Cw(n - 1) + Cw(n)]/2 (first test fish analysis)

$\overline{Cw}$ = [Cw(n - 2) + Cw(n - 1) + Cw(n)]/3 (2nd or later test fish analysis)

$\overline{Cw}$: mean test substance concentration in test water for calculation of bioconcentration factor (μg/L)

Cw(n): test substance concentration in the nth test water analysis determined at the same time as test fish analysis (μg/L)

(2) Bioconcentration factor calculation

$$BCF = Cf / \overline{Cw}$$

BCF	:	bioconcentration factor
Cf	:	test substance concentration in test fish (value with FB subtracted) (ng/g)
$\overline{Cw}$	:	mean test substance concentration in test water for calculation of bioconcentration factor (μg/L)
FB	:	mean concentration of test substance in test fish before and after experiment in the control group or apparent (blank) concentration of test substance (ng/g)

(3) Mean of bioconcentration factor of m^{th} time

BCFm = (BCFa + BCFb)/n

BCFm : mean of bioconcentration factor of m^{th} time (No. of individuals or group 2(a,b))

BCFa,b : bioconcentration factor of each individual or group of m^{th} time

n : number of individuals or group analyzed in the m^{th} time

The mean of the bioconcentration factor for a measurement of no detection is not to be determined.

4.7.9 Method of confirming steady state

The steady state is determined by observing a 20% or less fluctuation of the results of the 3 bioconcentration factor measurements in a row in an interval or 48 h or longer. If the bioconcentration factor is less than 100 times, it is considered to have reached a steady state after 28 days even if the bioconcentration factor fluctuation is over 20%.

Standard for attainment of a steady state: V(m - 2), V(m - 1), V(m) ≤ 20 (%)

$$V(m-2) = \frac{| BCF(m-2) - \overline{BCF} |}{\overline{BCF}} \times 100$$

$$V(m-1) = \frac{| BCF(m-1) - \overline{BCF} |}{\overline{BCF}} \times 100$$

$$V(m) = \frac{| BCF(m) - \overline{BCF} |}{\overline{BCF}} \times 100$$

V(m - 2), V(m - 1), V(m): variation (%) of bioconcentration factor from the mean

BCF(m - 2), BCF(m - 1), BCF(m): mean of the results of the $(m - 2)^{th}$, $(m - 1)^{th}$ and m^{th} bioconcentration factor of n individuals or groups

$\overline{BCF}$: {BCF (m - 2) + BCF (m - 1) + BCF (m)}/3

4.7.10 Method for calculation of steady state bioconcentration factor (BCFss)

The steady state bioconcentration factor (BCFss) was calculated using the following formulas.

(1) Calculation of mean test substance concentration in test water for calculation of steady state bioconcentration factor

$$\overline{Cws} = \{Cw(n-2) + Cw(n-1) + Cw(n)\} / 3$$

$\overline{Cws}$: mean test substance concentration in test water for calculation of steady state bioconcentration factor (as a rule, mean test substance concentration in test water measured 3 times in a row up to the final test fish analysis) (μg/L)

Cw(n): test substance concentration of n^{th} test water analysis determined simultaneously with test fish analysis (μg/L)

(2) Calculation of mean test substance concentration in test fish in a steady state

$$\overline{Cfs} = \{Cf(m-2) + Cf(m-1) + Cf(m)\} / 3$$

$\overline{Cfs}$: mean steady state test substance concentration in test fish (ng/g)

Cf(m): mean test substance concentration in m^{th} test fish analysis (value with FB subtracted) (ng/g)

FB: mean concentration of test substance in test fish before and after experiment in the control group or apparent (blank) concentration of test substance (ng/g)

(3) Calculation of steady state bioconcentration factor

$$BCFss = \overline{Cfs} / \overline{Cws}$$

BCFss: steady state bioconcentration factor

$\overline{Cfs}$: mean steady state substance concentration in test fish (ng/g)

$\overline{Cws}$: mean test substance concentration in test water for calculation of steady state bioconcentration factor (μg/L)

4.7.11 Calculatable bioconcentration factor

From the quantitative lower limit concentration of the test substances in test fish determined in section 4.7.6(4), it is possible to calculate a bioconcentration factor when it exceeds the following values. The test substance concentration in test water used was the mean test substance concentration in the case of all test water analyses.

Main component	First concentration level	5.1 times
	Second concentration level	51 times
Subcomponent	First concentration level	5.6 times
	Second concentration level	55 times

4.7.12 Method for calculation of fat content

The fat content was determined from the following formula.

Fat content (%) = $(T-T_0)/S \times 100$

T_0: weight of container (g)

T: weight of sample for weight analysis (including container) (g)

S: Sampled amount of pulverized test fish (g)

4.8 Handling of numbers

The method of JIS Z 8401: 1999 Rule B was used for rounding. The results used in calculation were not rounded afterward.

The test substance concentration in the test water and that in the test fish were rounded to 3 significant figures, and the bioconcentration factor was shown by rounding to 2 significant figures.

5. Environmental factors considered to affect reliability of test results

No corresponding factors were found.

6. Test results

6.1 Test substance concentration in test water

As apparent from the results shown in Table 1, the test substance concentrations in the test water were maintained above 88% of the values set. The fluctuations in test substance concentrations were maintained within ± 20% of the mean measurement result.

Table 1. Test substance concentration in test water

(Unit: μg/L)

Component	Concentration level	After 4 days	After 7 days	After 10 days	After 17 days	After 24 days	After 28 days	Mean (standard deviation)	Table	Fig
Main component	1	46.0	45.7	51.7	53.4	53.1	51.5	50.2 (3.47)	5	6
	2	4.43	48.7	5.08	5.47	5.27	4.75	4.98 (0.376)	6	
Subcomponent	1	45.7	45.3	51.7	46.5	50.7	48.4	48.1 (2.69)	5	
	2	4.39	5.01	5.20	5.17	5.20	4.56	4.92 (0.357)	6	

6.2 Bioconcentration factor

Table 2 shows the bioconcentration factor results.

The correlation between the bioconcentration factor results shown in Table 2 and exposure term is shown in Figure 1 and Figure 2. The bioconcentration results factor during the exposure term are as follows.

Main component	First concentration level	less than 5.1 times
	Second concentration level	less than 51 times
Subcomponent	First concentration level	350-510 times
	Second concentration level	270-430 times

Table 2. Bioconcentration factor

Mean inside ( )

Component	Concentration level	After 7 days	After 10 days	After 17 days	After 24 days	After 28 days	Table	Fig.
Main component	1	≦ 5.1 ≦ 5.1	≦ 5.1 ≦ 5.1	≦ 5.1 ≦ 5.1	≦ 5.1 ≦ 5.1	≦ 5.1 ≦ 5.1	8	9
	2	≦ 51 ≦ 51	≦ 51 ≦ 51	≦ 51 ≦ 51	≦ 51 ≦ 51	≦ 51 ≦ 51	9	10
Subcomponent	1	360 370 (370)	350 360 (350)	410 510 (460)	410 410 (410)	470 430 (450)	8	9
	2	380 320 (350)	370 320 (340)	430 340 (380)	350 340 (340)	270 270 (270)	9	10

6.3 Steady state bioconcentration factor

6.3.1 Main component

As a result of section 6.2, all of the final consecutive 3 analyses showed no detection, and consequently, no steady state bioconcentration factor could be calculated. However, the results on the bioconcentration factor were all less than 100 times, and thus, it was concluded that the steady state had been attained after 28 days.

6.3.2 Subcomponent

As a result of section 6.2, the results on the bioconcentration factor (mean) after 17, 24 and 28 days showed fluctuations within 20% of the mean bioconcentration factor of those 3 analyses, and consequently, it was concluded to have reached a steady state. Those results were used to calculate a steady state bioconcentration factor.

(1) Steady state test substance concentration in test water

As shown in Table 3, the mean test substance concentrations in test water in a steady state were 97% of the prescribed value in the first concentration level and 100% in the second concentration level.

Table 3. Steady state test substance concentration in test water

(Unit: μg/L)

Concentration level	After 17 days	After 24 days	After 28 days	Mean	Table	Figure
1	46.5	50.7	48.4	48.5	5, 8	6
2	5.17	5.20	4.56	4.98	6, 9	

(2) Steady state bioconcentration factor

The results on the steady state bioconcentration factor were as follows.

First concentration level 440 times

Second concentration level 340 times

6.4 Fat content of test fish

The results on the mean fat content of the test fish were as follows.

Before experiment 2.94%

After experiment 4.32%

6.5 Observed appearance of test fish

No abnormality was observed.

7. Discussion

The fluctuation in fat content (mean) after the experiment of the present study was 47% and exceeded 25% compared with the fat content before the experiment. The fluctuations in physiological conditions, feeding state, etc., are considered to be involved in the observed fat content fluctuation. The study on the feeding method, etc., will be continued for improvement with respect to fat content changes and fluctuations.

8. Notes

Major instruments, equipment, reagents, etc., used in the study were are as follows.

(1) Devices and equipment used in the study system (rearing facility)

Microquantitative pump for supplying stock solution:	GMW Model manufactured by Tokyo Rikakikai
Dissolved oxygen meter:	Model F-102 manufactured by Iijima Denshi Kogyo
pH meter:	Model HM - 14P manufactured by Toa Denpa Kogyo
Thermometer for water:	Model ALCO-50° manufactured by Andoh Keiki

(2) Instruments, devices, special equipment and reagents used for analysis and stock solution preparation

Instrument and equipment

Combined liquid chromatographer-mass spectrometer: see page 15

Balance: Model LP4200S manufactured by Sartorius
Model 1216MP manufactured by Sartorius
Model BP301S manufactured by Sartorius
Model PB602 manufactured by Mettler-Toledo

Fourier-transformed infrared spectrophotometer: Model FTIR-8200PC manufactured by Shimazu

Homogenizer (Polytron): Model PT3000 manufactured by Kinematica
Model PT3100 manufactured by Kinematica

Centrifuge: Model CR21G manufactured by Hitachi Koki

Pulverizer: stirring pulverizer manufactured by Ishikawa Kojo

Special equipment

SepPak C_{18}: manufactured by Nippon Waters

Reagents

Methanol: for HPLC manufactured by Wako Junyaku Kogyo

Formic acid: Grade 1 reagent manufactured by Wako Junyaku Kogyo

Ammonium acetate: Research grade reagent manufactured by Wako Junyaku Kogyo

Crystallized sugar: manufactured by Ohtori Hyoutou

Megafac F-142D: manufactured by Dainippon Ink Kagaku Kogyo

(3) Instruments, equipment and reagents used for fat content measurement

Balance: Model BP301S manufactured by Sartorius

Rotary evaporator: Model N - 1 manufactured by Tokyo Rika Kikai
Model N - 1000K manufactured by Tokyo Rika Kikai

Homogenizer (Polytron): Model PT3100 manufactured by Kinematica
Model PT3000 manufactured by Kinematica

Homogenizer (Autocellmaster):	Model CM - 200 manufactured by Iuchi Seieido
Vacuum pump:	Model DA-20D manufactured by Shinku Kiko
Vacuum desiccator:	Model VL manufactured by Iuchi Seieido
Reagents	
Purified water:	Pharmacopoeia japonica, manufactured by Takasugi Seiyaku
Methanol:	Grade 1 reagent, manufactured by Wako Junyaku Kogyo
Chloroform:	Research grade reagent manufactured by Wako Junyaku Kogyo
Sodium sulfate (anhydrous):	Grade 1 reagent, manufactured by Kanto Kagaku

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