FINAL REPORT

Bioconcentration Study of Hexabromocyclododecane in Carp

Conducted with 1,2,5,6,9,10-Hexabromocyclododecane (Test Substance No. K-1035)

Chemicals Inspection and Testing Institute
Chemical Biotesting Center, Kurume Laboratory

Compliance with the GLP Standards

Kurume Laboratory
Chemical Biotesting Center
Chemical Inspection and Testing Institute

Sponsor Ministry of International Trade and Industry

Study title Bioconcentration study of hexabromocyclododecane in carp conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

Study No. 51035 II

The study described in this report was conducted in compliance with the "Standard concerning testing facility provided in Article 4 of the Ordinance Prescribing Test Items Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances" (Kanpogyou No.39, Yakuhatsu No.229, 59 Kikyoku No.85, March 31, 1984; amended on November 18, 1988) and OECD Principles of Good Laboratory Practice (May 12, 1981).

Laboratory facility manager

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Sealed date: December 4, 1995

Quality Assurance Statement

Kurume Laboratory,
Chemical Biotesting Center
Chemical Inspection and Testing Institute

Sponsor Ministry of International Trade and Industry

Study title Bioconcentration study of hexabromocyclododecane in carp conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

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Above study was audited and inspected by the Quality Assurance Division of Kurume Laboratory, Chemical Biotesting Center, Chemical Inspection and Testing Institute.

The dates of study audit and inspection, and the dates of report of the results to the laboratory facility manager and study director are listed below.

Date of audit or inspection	Date of report to laboratory facility manager	Date of report to study director
November 2, 1992	November 2, 1992	November 2, 1992
November 17, 1992	November 24, 1992	November 24, 1992
November 18, 1992	November 24, 1992	November 24, 1992
December 14, 1992	December 17, 1992	December 17, 1992
December 15, 1992	December 17, 1992	December 17, 1992
November 17, 1995	November 17, 1995	November 17, 1995
December 4, 1995	December 4, 1995	December 4, 1995

I hereby certify that this report describes the precise methods of the study which was conducted in accordance with its protocol and with our standard operating procedures, and that the reported results reflect accurately the raw data of the study.

Quality Assurance Personnel

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Sealed date: December 4, 1995

Contents

Summary

1. Study title: Bioconcentration Study of hexabromocyclododecane in carp conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

2. Test conditions

2.1 Acute toxicity test

(1) Test fish: Medaka

(2) Exposure period: 48 hours

(3) Exposure regime: Semi-static (renewed every 8-16 hours)

2.2 Bioconcentration test

(1) Test fish: carp

(2) Test concentrations:

High concentration level	Component B	24 μg/L
	Component C	20.2 μg/L
	Component E	144 μg/L
Low concentration level	Component B	2.4 μg/L
	Component C	2.02 μg/L
	Component E	14.4 μg/L

(3) Exposure period: 14 weeks

(4) Exposure regime: Flow-through

(5) Analytical method: High-Performance Liquid Chromatography –Mass Spectrometry

3. Test results

(1) LC_{50} values after 48 hours: >250mg/L of supplied test substance

(2) Bioconcentration factor

High concentration level	Component B	834- 3070
	Component C	816- 1780
	Component E	118- 418
Low concentration level	Component B	3390-16100
	Component C	3350- 8950
	Component E	479- 2030

4. Stability of the test substance

Stability of the test substance under the storage and testing conditions was confirmed.

FINAL REPORT

Study No. 51035 II

1. Study title

Bioconcentration study of hexabromocyclododecane in carp conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

2. Sponsor

Ministry of International Trade and Industry

3. Testing facility

Name	: Chemical Inspection and Testing Institute Chemical Biotesting Center, Kurume Laboratory
Address	: 19-14 Chuo-cho, Kurume-shi, Fukuoka, Japan
Tel	: +81-942-34-1500
Laboratory facility manager	: **********

4. Purpose of the study

This study was conducted to evaluate bioconcentration potential of the test substance (K-1035).

5. Test method

This study was conducted in accordance with the Test Method Relating to New Chemical Substances "Bioconcentration test of chemical substances in fish and shellfish" (Kanpogyo No.5, Yakuhatsu No.615, 49 Kikyoku No.392, July 13, 1974), and OECD Guidelines for Testing of Chemicals 305C "Bioconcentration: Test for the degree of Bioconcentration in Fish" (May 12, 1981)

6. Compliance with GLP

This study was conducted in compliance with the "Standard concerning testing facility provided in Article 4 of the Ordinance Prescribing Test Items Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances" (Kanpogyou No.39, Yakuhatsu No.229, 59 Kikyoku No.85, March 31, 1984; amended on November 18, 1988) and OECD Principles of Good Laboratory Practice (May 12, 1981).

7. Test period

(1) Start of the test: November 2, 1992
(2) Start of the exposure: November 2, 1992
(3) End of the exposure: February 8, 1993
(4) End of the test: November 20. 1995

8. Testing personnel

Study director:	**********
Experimental scientists:	**********
	**********
	**********
	**********
	**********
Laboratory animal manager:	**********
Acute toxicity test experimental scientist:	**********
Storage division manager:	**********

9. Completion of the final report

November 20, 1995
Author: **********

10. Approval of the final report

November 20, 1995
Study director: **********

11. Test Substance

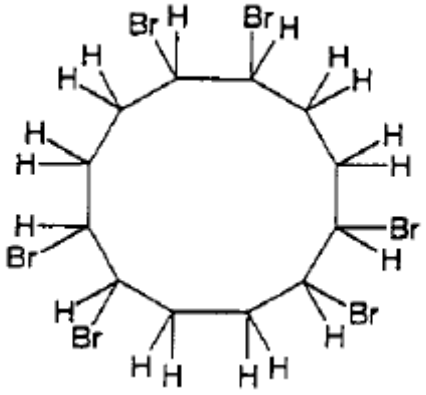
The test substance (K-1035) described herein is identified by following name, structure, etc.

11.1 Name

1,2,5,6,9,10-hexabromocyclododecane

11.2 Structure, etc. 6. Compliance with GLP

(1) Structural formula:



(2) Molecular formula: $C_{12}H_{18}Br_6$
(3) Molecular weight: 641.70

11.3 Purity

94.3%

As the supplied test substance was a mixture, it was separated by high performance liquid chromatography (HPLC) into 5 components which are referred to as components A-E according to the order of the peak appearance. Assuming that molar extinction coefficient of each component is equal, relative proportion of each component was calculated from its peak area relative to the total peak area. The results are shown as follows:

Component	Relative proportion (%)	Molecular formula
A	4.2	Unknown
B	12.0	$C_{12}H_{18}Br_6$
C	10.1	$C_{12}H_{18}Br_6$
D	1.5	Unknown
E	72.2	$C_{12}H_{18}Br_6$

It was confirmed by mass spectrometry (MS) that molecular formula of the components B, C and E are the same as that of the test substance. Therefore, analyses were made on components B, C and E in this study.

11.4 Supplier, commercial name, grade and lot number of the test substance[*1]

(1) Supplier: **********
(2) Commercial name: **********
(3) Grade: **********
(4) Lot No.: AN01
*1 information provided by the supplier

11.5 Identification of the test substance

Structure of the test substance was identified with infrared spectroscopy (see Fig. 14), mass spectrometry (see Fig. 15) and nuclear magnetic resonance spectroscopy (see Fig. 16).

11.6 Storage conditions and stability under those conditions

(1) Storage conditions: Cool and dark place
(2) Stability: Stability of the test substance was confirmed by infrared spectra measured before and after the exposure (see Fig.14).

11.7 Stability under the testing conditions

Stability of the test substance was confirmed by preliminary analysis performed prior to the start of exposure.

12. Acute toxicity test

As the test substance supplied for this study was the same as that used for study No. 51035, the result of the study No. 51035 was adopted.

12.1 Test method

The test was conducted according the method described in Japanese Industrial Standard (JIS K 0102-1986-71). "Testing methods for industrial waste water: Acute toxicity to fish"

12.2 Test fish

(1) Fish species: Japanese medaka (*Oryzias latipes*)
(2) Source: Nakajima Yougyojo
(Daimyoujin, Nagasumachi, Tamana-gun, Kumamoto, Japan)
(3) Quarantine conditions:
Period: Abnormal fish were removed by visual inspection upon their arrival. Remaining fish were treated with medicated bath and then cultured for 7 days under flow-through conditions
Medicated bath: Fish were treated with medicated bath containing 20mg/L Erubaju (Ueno Fine Chemicals Industry, Ltd.) and 7g/L sodium chloride solutions for 24 hours under static conditions.
(4) Acclimatization conditions:
Fish were acclimated in acclimatization vessel for 34 days under flow-through conditions at 25±2℃, and fish showing signs of abnormality were removed during this period.
(5) Body weight: average 0.21g
(6) Body length: average 3.0cm
(7) Screening: Fish from the same lot (TFO-900523) as the ones that passed the test with mercuric chloride were used for this study, according to the method described by Kenji Tabata[*2].

*2 Yosui to Haisui , 14, 1297-1303 (1972)

12.3 Dilution water

(1) Type
Ground water pumped up in the premise of the Kurume Laboratory
(2) Water quality
Temperature, pH and dissolved oxygen of the dilution water were regularly measured at Kurume Laboratory. Total hardness, evaporated residue, chemical oxygen demand, free chlorine and ammonia nitrogen as well as hazardous substances such as organic phosphorus, cyanide ions and heavy metals were measured periodically at 6 months intervals. Where this water was used for study, it was confirmed that total hardness and evaporated residue were below the criteria listed in the “Drinking Water Quality Standards based on Waterworks Act” (Kouseishourei No.56, August 31, 1978), and other items analyzed were below the concentrations specified in “Standards of Fishery Water” (Japan Fisheries Resource Conservation Association, March 1973) (see Appendix I).

12.4 Test conditions

(1) Test vessels:	Round glass aquaria
(2) Test solution volume:	4L / concentration
(3) Test solution temperature:	25±2℃
(4) Dissolved oxygen concentration:	at the start of the exposure 7.5mg/L
	at the end of the exposure 6.4mg/L
(5) pH:	at the start of the exposure 7.4
	at the end of the exposure 7.2
(6) Number of test fish:	10 fish / concentration
(7) Exposure period:	48 hours
(8) Exposure regime:	Semi-static (renewed at 8-16 hr. intervals)

12.5 Preparation of stock solution

(1) Dispersants
Crystal sugar and HCO-40
(2) Method of preparation
Supplied sample of the test substance was dissolved into deionized water after stone-milling with 20-fold crystal sugar and 30-fold HCO-40 to prepare a 500mg/L stock solution.

12.6 Conduct of the test

(1) Site: LC_{50} measurement room
(2) Dates: July 2-4, 1990

12.7 Calculation of 48 hr. LC_{50}

48 hr. LC_{50} was estimated by Doudoroff method.

12.8 Test result

An estimated 48 hr. LC_{50} value was above 250mg/L of the supplied sample (see Fig.3).

13. Bioconcentration test

13.1 Test fish

(1) Fish species: carp (*Cyprinus carpio*)
(2) Supplier: Sugishima Yougyojo (123-2 Gunchikuichibancho, Yatsushiro-shi, Kumamoto)
Received on September 8, 1992
(3) Quarantine conditions:
Period: Abnormal fish were removed by visual inspection upon their arrival. Remaining fish were treated with medicated bath and then cultured for 1 day under flow-through conditions
Medicated bath: Fish were treated with medicated bath containing 50mg/L terramycin for fish (Taito Phizer) and 7g/L sodium chloride solutions for 24 hours under static conditions.
(4) Acclimatization conditions:
Fish were acclimated in acclimatization vessel for 25 days under flow-through conditions at 25±2℃, and fish showing signs of abnormality were removed during this period. Then, the fish were transferred to test vessels and cultured for 23 days at the same temperature under flow-through conditions.
End of acclimatization: October 9, 1992
(5) Body weight, length[*3], etc. before the exposure
Body weight: average 22.0g
Body length: average 9.3cm
Lipid content: average 3.9%
*3 Measured value on lot No. TFC-920908

(6) Feed
Type: Mixed feed pellets for carp
Producer: Nippon Formula Feed Manufacturing Co. Ltd.
Feeding regime: Fish were fed twice daily at a total rate of approx. 2% of their body weight per day. Feeding was stopped on the day before sampling.

13.2 Dilution water

Same as section 12.3

13.3 Testing and environmental conditions

(1) Supply of the test solutions: Flow-through apparatus assembled at the Kurume laboratory was used.
(2) Test vessels: 100L glass aquaria
(3) Test solution volume: Feed solution (4mL/min.) and dilution water (800mL/min.) were mixed and supplied to test vessels at a rate of 1158L/day.
(4) Test temperature: 25±2℃
(5) Dissolved oxygen concentration
High concentration level: 6.1-7.6mg/L (see Fig.11)
Low concentration level: 6.3-7.7mg/L (see Fig.12)
Control: 7.0-7.8mg/L (see Fig.13)

(6) Number of test fish
High/low concentration level: 20 (at the start of the exposure)
Control: 5 (at the start of the exposure)
(7) Exposure period: 14 weeks
(8) Experimental site: Aquatron room 213

13.4 Preparation of feed solutions

(1) Dispersants
Same as section 12.5 (1)
(2) Method of preparation
- High concentration level
Supplied sample (1mg) and crystal sugar (20g) were dissolved in deionized water after stone-milling with 30g of HCO-40, and then transferred to a 25L glass feed solution tank and filled with deionized water to be supplied to the test vessel.
- Low concentration level
Supplied sample (0.1mg) and crystal sugar (2g) were dissolved in deionized water after stone-milling with 30g of HCO-40, and then transferred to a 25L glass feed solution tank and filled with deionized water to be supplied to the test vessel.
- Control
Crystal sugar (20g) was dissolved in deionized water after stone-milling with 30g of HCO-40, and then transferred to a 25L glass feed solution tank and filled with deionized water to be supplied to the control vessel.

13.5 Test concentrations

Test concentrations were set as follows by considering 48 hr. LC_{50} value and analytical sensitivity of each component:

Unit: μg/L

	Supplied sample	Components				
		A	B	C	D	E
High concentration	200	8.4	24	20.2	3	144
Low concentration	20	0.84	2.4	2.02	0.3	14.4

Negative control was also set up.

13.6 Analysis of the test substance in test solutions and test fish

Analyses of the test substance were performed for components B, C and E with consideration to analytical sensitivity.

13.6.1 Frequency of analysis

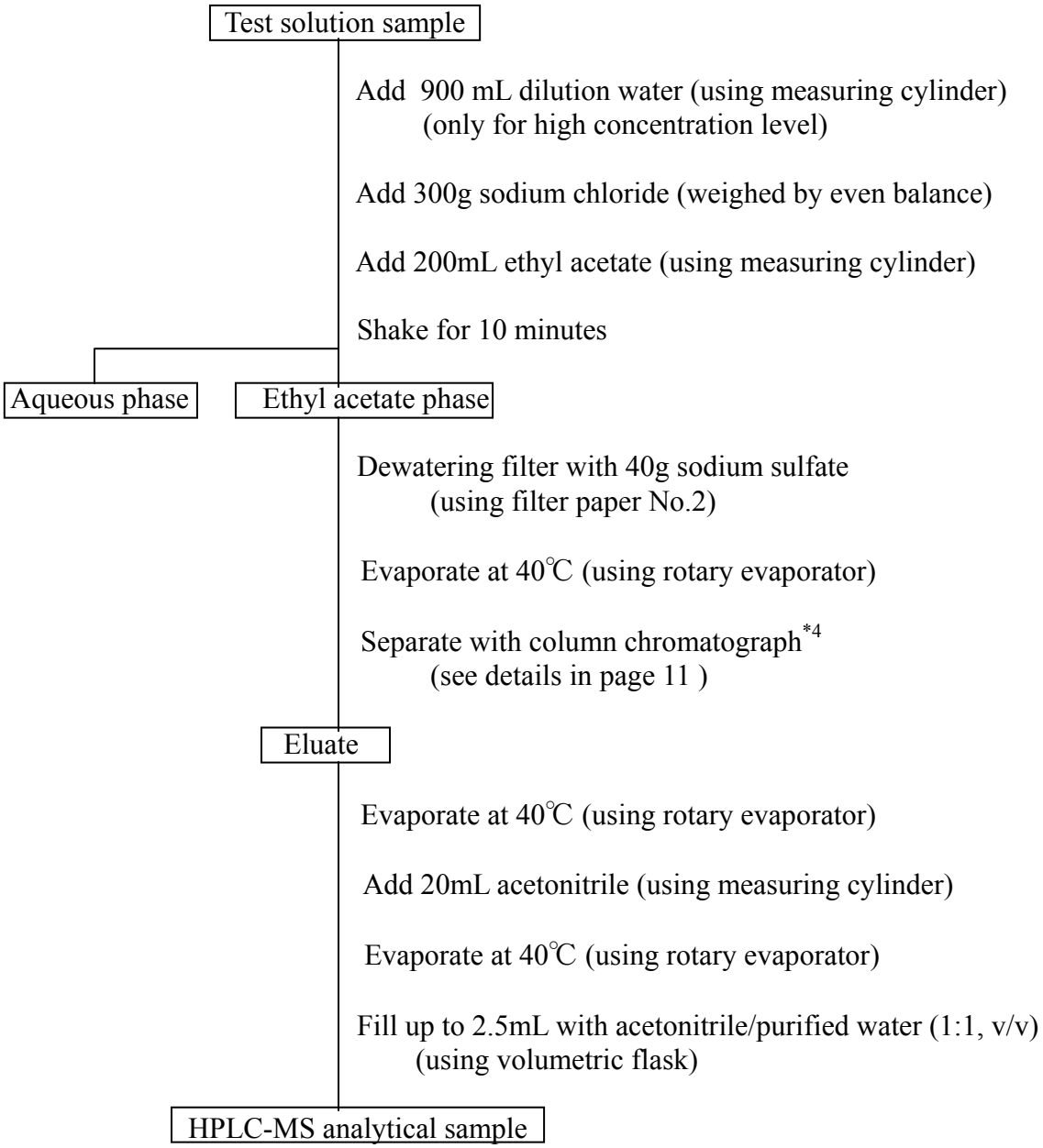
Analyses of the test substance in test solutions were performed twice weekly during the exposure period for both high and low concentrations with one sample each per analysis. Analyses of the test substance in fish were performed 7 times at 2, 4, 6, 8, 10, 12 and 14 weeks after the start of the exposure for both high and low concentrations with two samples each per

analysis. Analyses of the test substance in control fish were performed at the start and end of the exposure with 2 samples per analysis.

13.6.2 Pre-treatment of analytical samples

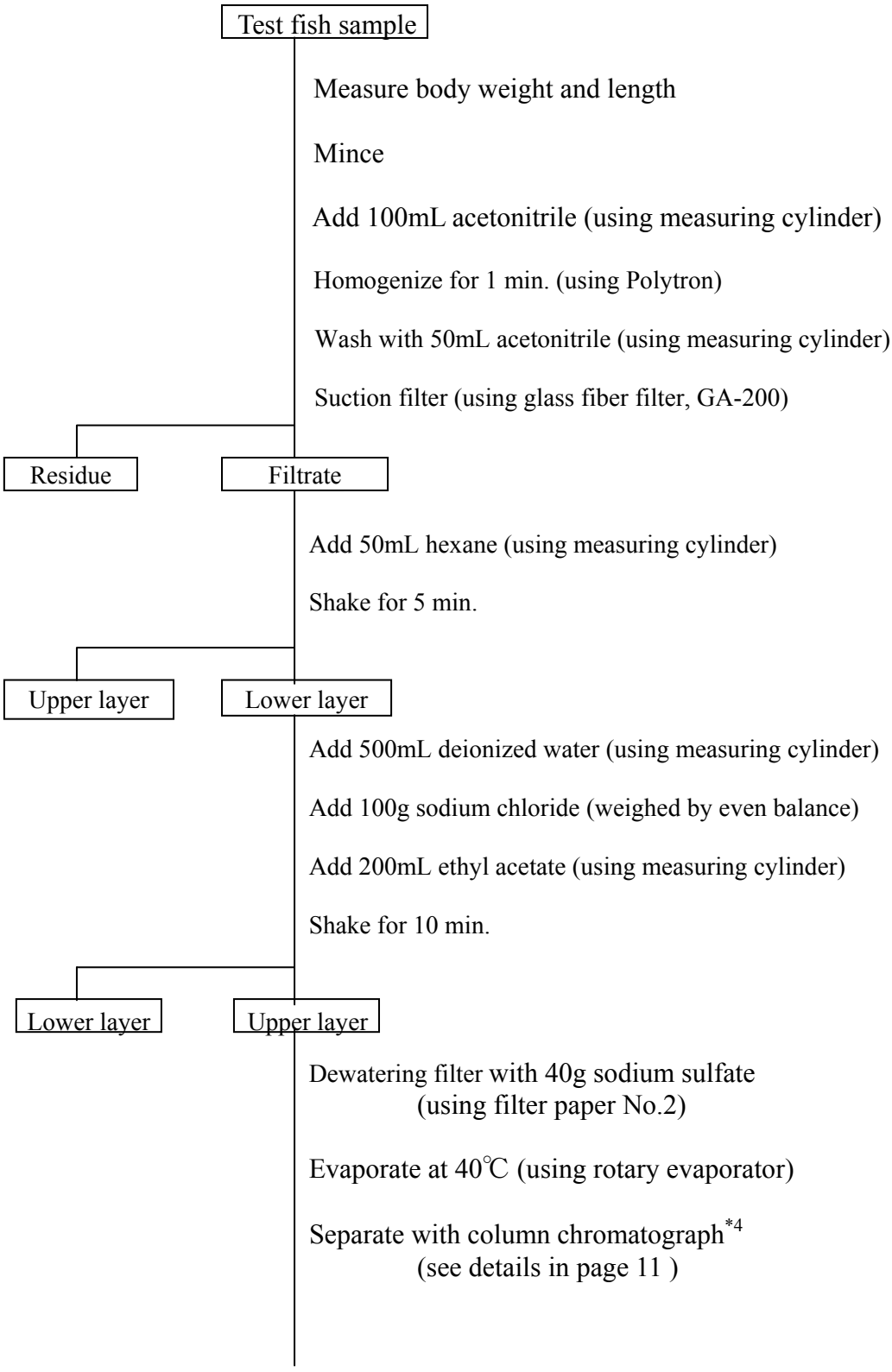
(1) Test solutions

Samples of test solutions (100mL and 1000mL for high and low concentrations, respectively) were taken from test vessels and pre-treated according to the procedure described in the following flow chart to prepare samples for HPLC-MS analysis:



(2) Test fish

Test fish were sampled from the test vessels and pre-treated according to the procedure described in following flow chart to prepare samples for HPLC-MS analysis：



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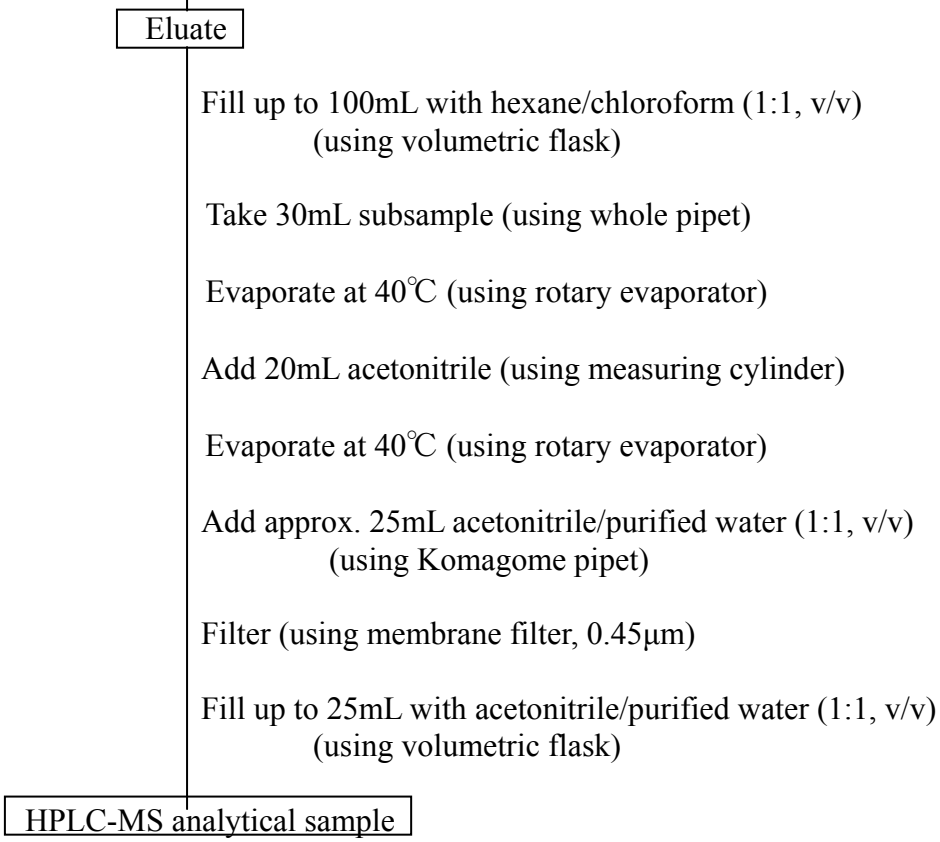

*4 Column chromatographic conditions

Column:	20mm ϕ glass tube
Column packing material:	5% hydrous basic aluminum 5g (filled with hexane)
Loading solvent:	Hexane 10mL
Eluting solvents:	1. hexane 30mL (including portions used for loading)
	2. hexane/chloroform (1:1,v/v) 60mL
	Components B, C and E were eluted with the second eluent.

13.6.3 Quantitative analysis of the test substance

The test substance in samples prepared by the procedure described in section 13.6.2 was quantified by HPLC-MS analysis. Fish samples for HPLC-MS analysis were diluted as appropriate in order to adjust concentrations to fall within the range showing linear correlation. Concentration of the test substance in final samples were calculated from peak area on mass fragmentgram relative to peak area of standard solution of known concentration (see Tables 4, 5, 8-10 and Figures 6, 8-10).

13.6.3.1 Analytical conditions

(1) HPLC

Apparatus: High performance liquid chromatograph

Eluting conditions

Column: L-column ODS
15cm × 4.6mmφ stainless steel

Mobile phase: acetonitrile/purified water (85:15, v/v)

Flow rate: 1.0mL/min

Injection volume: 100μL

Matrix conditions

Matrix solution : 0.5 or 1% glycerine/acetonitrile

Flow rate: 0.5mL/min.

(2) MS

Apparatus: gas chromatograph-mass spectrometer

Split conditions

Split method: air pressure splitter

Split pressure: 1.3kg/cm^3

Mass spectrometric conditions

Monitoring ion: negative ion

Measured mz: 721.6

Ionizing method: Frit-FAB method

Ionizing gas: xenon

13.6.3.2 Preparation of standard solutions

Standard solutions used for quantification of the test substance in analytical samples were prepared as follows:

A 100mg portion of supplied test substance was accurately weighed and dissolved in acetonitrile to make a 1000μg/mL stock solution. A 8μg/mL standard solution of supplied test substance was made from this stock solution by diluting with acetonitrile/purified water (1:1, v/v). Concentrations of the components are as shown below.

Unit: μg/mL

Components		
B	C	E
0.96	0.808	5.78

13.6.3.3 Construction of calibration curves

Standard solutions at concentrations of 4, 8 and 16μg/mL of supplied test substance were prepared by the same procedure as described in section 13.6.3.2. Concentrations of the components are as shown below.

Unit: μg/mL

Supplied sample	Components		
	B	C	E
4	0.48	0.404	2.89
8	0.96	0.808	5.78
16	1.92	1.62	11.6

These standard solutions were then analyzed under the conditions described in section 13.6.3.1. Peak areas of each component on mass fragmentgrams were plotted against concentrations in standard solutions to draw a calibration curve.

Limits of quantification (LOQ) of the test substance were determined from the calibration curves with considerations to noise levels to be as follows:

(1) Recovery test, blank test, analysis of test solutions at days 4-9 and analysis of control fish before the start of exposure

Component	Peak area	Concentration (μg/mL)
B	6	0.037
C	7	0.024
E	30	0.15

(2) Analysis of test solutions at days 12-96 and analysis of test fish at weeks 2-14 and control fish at the end of exposure

Component	Peak area	Concentration (μg/mL)
B	6	0.047
C	7	0.027
E	30	0.17

13.6.4 Recovery test and blank test

(1) Method

Recovery test was performed to determine recovery rate of the test substance in pre-treatment procedure used to prepare analytical samples of test solutions and fish as described in section 13.6.2, by analyzing test solution and fish homogeneate spiked with the test substance. Blank test was also performed by analyzing test solution and fish homogeneate with no spiked test substance. Recovery and blank tests were both performed on duplicate samples. No peak on mass fragmentgram was observed at identical position to the test substance in the blank test. Recovery rates of duplicate samples and average values are shown below. The average recovery rates were used to correct concentrations of the test substance in analytical samples (see Tables 3, 7, Figures 5 and 7).

(2) Results

Recovery rates from analytical procedure (%)

Test solution

Component B	79.6	75.2	Av. 77.8
Component C	82.7	70.8	Av. 76.8
Component E	98.5	92.2	Av. 95.4

Test fish

Component B	94.8	95.6	Av. 95.2
Component C	89.1	99.6	Av. 94.4
Component E	103	89.9	Av. 96.4

Spiked test substance (μg)

	Supplied sample	Components		
		B	C	E
Test solution	20	2.4	2.02	14.4
Test fish	600	72	60.6	433

13.6.5 Calculation of test substance concentrations in analytical samples

13.6.5.1 Calculation of test substance concentrations in analytical samples of the test solutions

Calculation was done according to the formula in Table 6. Calculated results are presented in 3 significant figures.

13.6.5.2 LOQ of the test substance in test solutions

LOQs of the test substance in test solutions were determined from the LOQs determined in section 13.6.3.3 to be as follows:

Days	Concentration level	Component	LOQ (μg/mL)
4-9	High	B	0.0012
		C	0.00079
		E	0.0039
	Low	B	0.00012
		C	0.000079
		E	0.00039
12-96	High	B	0.0015
		C	0.00088
		E	0.0045
	Low	B	0.00015
		C	0.000088
		E	0.00045

13.6.5.3 Calculation of test substance concentrations in analytical samples of the test fish

Calculation was done according to the formula in Table 11. Calculated results are presented in 3 significant figures.

13.6.5.4 LOQs of the test substance in test fish

By assuming fish body weight to be 30g, LOQs of the test substance [*5] in test fish were determined from the LOQs determined in section 13.6.3.3 to be 0.14, 0.079 and 0.49 μg/g for components B, C and E, respectively.

*5 $\text{LOQ of the test substance } (\mu\text{g/mL or } \mu\text{g/g}) = \dfrac{\text{A}}{\dfrac{\text{B}}{100} \times \dfrac{\text{C} \times \text{E}}{\text{D}}}$

where
A: LOQ concentration on the calibration curve (μg/mL)
B: Recovery rate (%)
C: Test solution volume (mL) or test fish body weight (g)
D: Final volume of the sample (mL)
E: Preparative ratio

Calculated results are presented in 2 significant figures.

13.7 Calculation of bioconcentration factors (BCF)

Calculation was done according to the formula in Table 11. Calculated results are presented in 3 significant figures.

Calculation of bioconcentration factor is possible when it exceeds following limits derived from the LOQs of the test substance in test fish determined in section 13.6.5.4

Concentration level	Component	BCF
High	B	6.1
	C	4.1
	E	3.5
Low	B	71
	C	49
	E	36

13.8 Treatment of the values

Values were rounded according to the standard described in JIS Z 8401-1961.

14. Test results

14.1 Test substance concentration in test solutions

Concentrations of the test substance in test solutions are shown in Table 1.

Table 1 Test substance concentrations (μg/L) in test solutions
(average measured values after the start of exposure)

Conc. level	Compo-nent	Weeks							Attached table	Attached figure
		2	4	6	8	10	12	14		
High	B	22.4	21.8	21.8	22.2	22.4	22.4	22.9	Table 4	Figure 6
	C	19.0	19.2	19.4	19.5	19.4	19.1	19.5		
	E	140	138	141	142	141	138	139		
Low	B	1.96	1.79	1.72	1.79	1.86	1.91	1.98	Table 5	
	C	1.55	1.46	1.41	1.48	1.53	1.54	1.61		
	E	14.1	13.6	13.3	13.5	13.4	13.3	13.5		

14.2 BCF

BCFs are shown in Table 2.

Table 2 Bioconcentration factors

Conc. level	Compo-nent	Weeks							Attached table	Attached figure
		2	4	6	8	10	12	14		
High	B	834 965	1550 1470	1650 1970	2650 1580	1640 2470	3070 2150	2040 2660	Table 8	Figure 8
	C	816 898	1410 972	850 1250	1480 1780	837 1670	1460 1290	1200 1440		
	E	123 143	182 138	118 168	260 300	200 307	418 243	202 306		
Low	B	3810 3840	3390 6870	11900 11000	11700 6880	12000 12400	9900 12700	16100 15200	Table 9	Figure 9
	C	3370 3350	3740 5720	7090 7400	7870 8360	5060 6020	4520 6460	7860 8950		
	E	589 601	479 955	1120 996	1290 890	1510 1470	1370 1800	2030 1990		

Correlation between BCF and exposure duration in Table 2 is shown in attached Figures 1 and 2. From these figures, it can be considered that steady state had been reached by 14 weeks. The degrees of bioconcentration potential of the test substance in carp in terms of BCF are as follows:

Concentration level	Component	BCF
High	B	834 - 3070
	C	816 - 1780
	E	118 - 418
Low	B	3390 - 16100
	C	3350 - 8950
	E	479 - 2030

14.3 Observation of the test fish

No sign of abnormality was observed.

15. Referential studies

15.1 Purpose

Tissue-specific bioconcentration and elimination of the test substance in and from the test fish were studied.

15.2 Tissue-specific bioconcentration

15.2.1 Method

Test fish sampled from both high and low concentration levels at week 14 were separated into parts such as integument (skins except for head skin, fins, digestive tract and gills), head, viscera (except for digestive tract) and fillets. Concentrations of the test substance in analytical samples of each part were determined to calculate tissue-specific bioconcentration factor.

15.2.2 Results

Calculation was done according to the formula in Table 11. Calculated values are presented in 3 significant figures.

The results are shown below.

Conc. level	Compo-nent	Part	Test substance concentration (μg/L)	Concentration factor	Attached table	Attached figure
High	B	Fillet	37.3	1630	Table 12	Figure 17
		Head	127	5540		
		Integument	101	4390		
		Viscera	135	5900		
	C	Fillet	22.6	1160		
		Head	66.8	3430		
		Integument	53.4	2740		
		Viscera	80.7	4140		
	E	Fillet	29.2	210		
		Head	91.9	661		
		Integument	68.3	491		
		Viscera	102	735		
Low	B	Fillet	6.85	3460		
		Head	54.4	27500		
		Integument	30.2	15300		
		Viscera	16.6	8380		
	C	Fillet	3.22	2000		
		Head	33.2	20600		
		Integument	10.4	6430		
		Viscera	4.90	3050		
	E	Fillet	5.18	383		
		Head	43.4	3220		
		Integument	21.5	1590		
		Viscera	10.9	810		

15.3 Elimination

15.3.1 Method

Fish from both high and low concentration levels were transferred to test vessels supplied with test water containing no test substance or dispersant at week 14 (end of exposure and start of elimination). Fish were analyzed at 14, 28 and 56 days after the start of the elimination period according to the procedure described in section13.6.

Test vessels: 100L glass aquaria
Water supply: 1152L/ day at a rate of 800mL.min/
Temperature: 25 ±2℃

15.3.2 Residual rate of the test substance in test fish

Residual rates of the test substance in test fish were calculated by following equation and calculated results are presented in 3 significant figures.

$$PFn = \frac{\frac{Fn}{W}}{CF_{14}} \times 100$$

Where

PFn: Residual rate of the test substance in test fish after n days (%)
Fn: Absolute amount of the test substance contained in test fish after n days (μg)
W: Body weight of the fish (g)
CF_{14}: Average concentration of the test substance in test fish at 14 weeks after the start of exposure (μg/g)

15.3.3 Results

Concentrations of the test substance and the residual rates calculated in 1.3.2 are shown below (see attached Tables 14-15 and Figure 19-20). Correlation between residual rate and elimination period are shown in attached Figure 21.

Conc. level	Com-ponent	14 days		28 days		56 days	
		Test subs. conc.(μg/g)	Residual rate (%)	Test subs. conc.(μg/g)	Residual rate (%)	Test subs. conc.(μg/g)	Residual rate (%)
High	B	40.2	74.7	42.6 28.2	79.2 52.4	-	-
	C	10.2	39.7	6.75 2.16	26.3 8.4	1.69 3.10	6.6 12.1
	E	33.1	94.0	12.3 6.11	34.9 17.4	6.38 7.49	18.1 21.3
Low	B	29.7	95.8	33.1 19.6	107 63.2	10.4	33.5
	C	6.65	48.9	6.05 0.767	44.5 5.6	-	-
	E	12.7	46.9	12.8 8.37	47.2 30.9	1.81	6.7

Half lives ($t_{1/2}$) were calculated to be as follow:

Concentration level	Component	Half-life (days)
High	B	38.6
	C	16.2
	E	22.6
Low	B	38.3
	C	10.5
	E	15.2

16. Discussion

Quantification of component C was not possible in the previous study (Test No. 51035) due to insignificant difference from the test solution or fish blank. HPLC-MS method employed in this study enabled high sensitivity analysis, and the nominal concentrations in this test were set at 1/5 of those used in the previous study. Bioconcentration factors were calculated for components B, C and E, and the results indicate dose-dependency for all of those components.

17. Treatment of the values

Values were rounded according to the standard described in JIS Z 8401-1961.

18. Environmental factors that might have affected the reliability of the test results

There were no such factors.

19. Storage and retention of samples and records

19.1 Test substance

A portion of the test substance (5g) was placed in a storage container, tightly sealed and stored at the sample storage room of the Kurume Laboratory for as long as it can be stably stored.

19.2 Raw data, records, etc.

Results of analyses, measurements and observations generated in the study and other raw data such as laboratory notebooks which were used for the development of final report, test protocol, instructions, reference materials, etc. are stored along with the final report in the archive of the Kurume Laboratory until otherwise notified by the sponsor.

20. Major apparatuses

20.1 Apparatuses in the test system (culturing facility)

Micro liquid dosing pump for supplying feed solution:
Tokyo Rikakikai Co., Ltd. G.MW
Dissolved oxygen meter: Iijima Electronics Corporation 552

20.2 Apparatuses, instruments and reagents used for the analysis of samples and preparation of test solutions

20.2.1 Apparatuses and instruments

HPLC:	see page 8
GC-MS:	see page 9
Rotary evaporator:	Tokyo Rikakikai Co., Ltd. N-1
Shaker:	Irie Shokai Co., Ltd. TS
	TAITEC Co., Ltd. SR-II W
Homogenizer (Polytron):	Kinematica Inc.

20.2.2 Reagents

Sodium chloride:	Manac Inc. reagent grade
Ethyl acetate:	Kanto Chemical Co. Inc. reagent grade
Sodium sulfate anhydrous:	Katayama Chemical, Ltd. reagent grade
Acetonitrile:	Katayama Chemical, Ltd. reagent grade
	Wako Pure Chemical Industries, Ltd. HPLC grade
Hexane:	Wako Pure Chemical Industries, Ltd. reagent grade
Chloroform:	Kishida Chemical Co., Ltd. analytical reagent grade
Glycerine:	Nacalai Tesque, Inc. analytical reagent grade
Crystal sugar:	Mitsuibishi Corporation
HCO-40:	Nikko Chemicals Co., Ltd.
Purified water:	Takasugi Pharmaceuticals Co., Ltd. Japanese Phamacopeia
Basic aluminum:	INC Biomedical