CERI

Receipt number	662-16-E-7726
Study number	97726

FINAL REPORT

A 48-hour Acute Immobilization Study of APFHx (C-1500N) in *Daphnia magna*

This is a correct copy of the original.
Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)

Date June 2, 2017

Study Director Ryuta Adachi

June, 2017

Chemicals Evaluation and Research Institute, Japan, Kurume

GLP STATEMENT

Chemicals Evaluation and
Research Institute, Japan, Kurume

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of APFHx (C-1500N) in *Daphnia magna*

Study number 97726

The study described in this report was conducted in compliance with the following GLP principle:
"OECD Principles of Good Laboratory Practice" November 26, 1997, ENV/MC/CHEM (98)17

This final report reflects the raw data accurately and it has been confirmed that the test data are valid.

Date June 2, 2017

Study Director Ryuta Adachi
Ryuta Adachi

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Kurume

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: A 48-hour Acute Immobilization Study of APFHx (C-1500N) in *Daphnia magna*

Study number: 97726

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

Item of inspection	Date of inspection	Date of report
Study plan	April 19, 2017	April 19, 2017
Start of exposure	April 24, 2017	April 24, 2017
Completion of exposure	April 26, 2017	April 26, 2017
Raw data and draft final report	June 2, 2017	June 2, 2017
Final report	June 2, 2017	June 2, 2017

Date June 2, 2017

Personnel of Quality Assurance Unit: Toshiyuki Mitsunaga

Toshiyuki Mitsunaga

CONTENTS

Tables

1. Title

A 48-hour Acute Immobilization Study of APFHx (C-1500N) in *Daphnia magna*

2. Sponsor

Name DAIKIN INDUSTRIES, LTD.
Address 1-1, Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. Test facility

Name Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)
Address 3-2-7, Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan

4. Objective

The objective of this study is to determine the 48-hour median effective concentration (EC_{50}) by conducting an acute immobilization study of the APFHx (C-1500N) in *Daphnia magna*.

5. Test method

OECD Guidelines for Testing of Chemicals, No.202, April 13, 2004, "*Daphnia* sp., Acute Immobilisation Test"

6. GLP principle

"OECD Principles of Good Laboratory Practice" November 26, 1997, ENV/MC/CHEM (98)17

7. Dates

Study initiation date	April 18, 2017
Experimental starting date	April 24, 2017
Experimental completion date	April 26, 2017
Study completion date	June 2, 2017

8. Storage of test item, raw data, etc.

The study plan (original), the final report (original), the raw data, documents concerning the study presented by the sponsor, the test sample survey sheets and other reports are stored in the archives of this laboratory. The test item is returned to the sponsor.

The storage period is 10 years after the study completion date.

Treatment of the raw data, etc. after the storage period (continue, reject, or return) is discussed with the sponsor.

9. Personnel

Study Director	Ryuta Adachi (Section 4)
Study personnel (Biological study)	Naohiro Mizoguchi
Study personnel (Analytical chemistry)	Mika Ono

10. Approval of final report

Date June 2, 2017

Study Director Ryuta Adachi

Ryuta Adachi

11. Summary

Test item

APFHx (C-1500N)

Objective

The objective of this study is to determine the 48-hour median effective concentration (EC_{50}) by conducting an acute immobilization study of the APFHx (C-1500N) in *Daphnia magna*.

Test method

OECD Guidelines for Testing of Chemicals, No.202, April 13, 2004, "*Daphnia* sp., Acute Immobilisation Test"

Test conditions

Test organism	*Daphnia magna*
Dilution water	Dechlorinated tap water
Test level	100 mg/L and a control
Preparation of test solution	Test sample and dilution water were mixed and stirred to prepare the nominal concentration of 100 mg/L as the test solution.
Type of test	Static regime
Exposure duration	48 hours
Replicate	4 replicates/test level
Number of organism	20 daphnids/test level (5 daphnids/test vessel)
Volume of test solution	400 mL/test level (100 mL/test vessel)
Temperature of test solutions	20.3-20.4°C
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	No feeding
Aeration	No aeration
Analysis of concentration of test item in test solution	HPLC analysis (at the start and end of the exposure)

Results

48-hour EC_{50}	>100 mg/L

(The above-described value was based on nominal concentration.)

12. Test materials

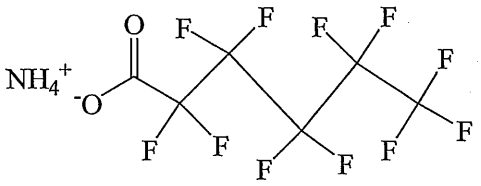
12.1 Test item

a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Another name	APFHx (C-1500N)
CAS number	21615-47-4

b) Chemical structure etc.

Structural formula



Molecular formula	$C_6H_4F_{11}NO_2$
Molecular weight	331.08

c) Test sample

Purity of test item	50%
Impurity	Water 50%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150E62004

The test item was treated with correcting by the purity of the test item.

d) Physicochemical properties

Appearance	Colorless and clear liquid

e) Storage condition

The test sample was stored in a dark storage place at room temperature.

f) Identification and stability of test item under the storage condition

The infrared (IR) spectrum of the test item measured at this laboratory was confirmed to be identical to that provided by the sponsor.

The stability of the test item was confirmed by comparing the IR spectrum of the test item after the completion of the experiment under the storage condition with that before the start of the experiment.

g) Safety and handling

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, mask, safety glasses, and white coats were worn when handling test item.

12.2 Test organisms

Species *Daphnia magna* Clone A

Reason for selection of species Species recommended in the test guideline

Source of supply The University of Sheffield, UK

Date of supply July 9, 1990

Acclimation Young daphnids produced by parents that were cultured in this laboratory were used. The parents to obtain young daphnids were bred in the same quality of water (dechlorinated tap water), water temperature (20±1°C) and photoperiod (16-hour light/8-hour dark) as used in the test. The parent animals used for the test were same lot and their age and survival rate were 20-day old and 100%, respectively. *Chlorella vulgaris* of 0.1-0.2 mgC (Organic carbon content)/day per *Daphnia* was fed to the parents once a day.

Selection of young daphnids Less than 24-hour-old daphnids

Allocation to the test groups Random sampling

Confirmation of reproducibility of test system A 48-hour acute immobilization test of a reference substance with the test organisms was periodically conducted. The latest data is shown below.

Reference substance Potassium dichromate (JIS special grade, Wako Pure Chemical Industries, Ltd., Lot No. JPJ7565)

Period of study April 4-April 6, 2017

48-hour EC_{50} 0.15 mg/L

This value was within the stipulated range (mean ± 2S.D.) [mean ± S.D.: 0.23±0.06 mg/L (n=100)] to background data in this laboratory.

13. Test methods

13.1 Dilution water

Dechlorinated tap water, aerated sufficiently and temperature-controlled, was used. Some chemical characteristics of the dilution water measured regularly are listed in Appendix 1. The result of chemical characteristics of the dilution water met the standards that were provided with the standard operation procedure of this laboratory.

13.2 Test apparatus and equipment

Test vessel 100 mL glass beaker

Cover of test vessel Transparent plastic lid

Water bath Plastic tank

Warming/cooling unit; Type HCA 250 (Sato craft)

13.3 Preparation of test solution

The test sample of 100 mg and dilution water of 0.5 L were mixed in preparation container and it was stirred to prepare the dissolved test solution. The test solution was divided into each test vessel.

13.4 Test conditions

Type of test	Static regime (no renewal of test solution)
Exposure duration	48 hours
Test concentration	100 mg/L (upper limit concentration of test method as a limit test) The test concentration was decided from the results of preliminary study. The results of the preliminary study are shown in Additional data.
Control	Dilution water without the test item
Replicate	4 replicates/test level
Number of organism	20 daphnids/test level (5 daphnids/test vessel)
Volume of test solution	400 mL/test level (100 mL/test vessel)
Temperature of test solution	20±1°C
Dissolved oxygen concentration	More than 3 mg/L without aeration
pH adjustment	No adjustment
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	No feeding

13.5 Observation and measurements

a) Observation of test organisms

Observation about immobility and symptoms of test organism was conducted at 24 and 48 hours after exposure. Daphnids were considered immobile if they were not able to swim within 15 seconds after gentle agitation of the test vessel.

b) Appearance of test solution

Observation at the start and end of exposure

c) Condition of test solutions

Item of measurement	Dissolved oxygen concentration, pH and temperature
Frequency of measurement	At the start and end of exposure
Sample for measurement	Another solution sampled separately from the preparation container (at the start of exposure) One test vessel in each test level (at the end of exposure)
Instrument	Dissolved oxygen meter YSI MODEL 58 (YSI Nanotech Japan) pH meter HM-21P (DKK-TOA) Thermometer of glass stick type

d) Concentration of test item in test solution

Frequency of measurement	At the start and end of exposure
Sample for measurement	Another solution sampled separately from the preparation container (at the start of exposure) The mixed solution taken out with equal volume from the middle layer of the test solution in test vessels in each test level (at the end of exposure)
Volume of sample	Approximately 10 mL (all test levels)
Analytical condition	Refer to Appendix 2

13.6 Calculating method of EC_{50}

Since more than 50% immobility could not be obtained in the exposure level, the EC_{50} value was estimated as "> test concentration".

The results of this study were estimated based on nominal concentrations since the measured concentration of test item in test solution were maintained within ±20% of the nominal concentrations during exposure.

13.7 Validity of test

a) The immobilization rate should not be more than 10% in control group during exposure.

b) Not more than 10% of the control daphnids should show the signs of disease or stress, for example, discoloration or unusual behavior such as trapping at surface of water.

c) Dissolved oxygen concentration should be more than 3 mg/L at the end of exposure.

13.8 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401: 1999 rule B.

(JIS; Japanese Industrial Standards)

14. Results and discussion

14.1 Immobility

Immobility at 24 and 48 hours is shown in Table 1.

No immobility was obtained in the exposure level during exposure. Immobility in the control was 0%, which met the criterion for the validity of the test (i.e. not more than 10%).

14.2 Observed abnormal response

The abnormal responses observed during exposure are shown in Table 2.

In the exposure level, neither immobilization nor abnormalities of behavior or appearance were observed.

In the control, no abnormal response (discolor of body, trapping at the surface of the water and so on), which met the criterion for the validity of the test (i.e. not more than 10%), was observed during exposure.

14.3 Observation and measurement of test solution

a) Appearance of test solution

The test solutions in the exposure level and the control were colorless and clear at the start and end of exposure.

b) Condition of test solutions

Condition of the test solutions is shown in Table 3.

The measured values of dissolved oxygen concentration, pH and temperature of the test solutions during exposure ranged from 8.8 to 8.9 mg/L, from 7.7 to 7.8 and from 20.3 to 20.4°C, respectively. The measured values of dissolved oxygen concentration met the criterion for the study validity (more than 3 mg/L at the end of exposure).

c) Concentration of test item in test solution

The method and result of the measured concentrations of the test item are shown in Appendix 2. Calibration curve and chromatograms are shown in Appendix 3.

The measured concentration of the test item in the test solution at the start of exposure was 102 mg/L (102% of the nominal concentration), and that at the end of exposure was 101 mg/L (101% of the nominal concentration). The measured concentrations of the test item were kept within ± 20% of the nominal concentration.

14.4 EC_{50}

The EC_{50}s at each observation time are shown in Table 4.

The 24-hour and 48-hour EC_{50}s of the test item for *Daphnia magna* were both >100 mg/L.

14.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at upper limit concentration of test method (100 mg/L). As a result, no adverse effect was found in the definitive study. Therefore, it was decided that the test item had no adverse acute effect on the test organisms at upper limit concentration of test method. The measured concentrations of the test item in the test solution were within the range of ±20% of the nominal concentration. The environmental conditions were within the suitable range; therefore, it is concluded that this study complied with the applied test guidelines.

15. Factors that affected the reliability of the test results

There were no factors which might have affected the reliability of the test.

Table 1 Immobility

Nominal concentration (mg/L)	Vessel	24 hours		48 hours	
		Number of immobilized daphnids/ Total daphnids	Immobility (%)	Number of immobilized daphnids/ Total daphnids	Immobility (%)
Control	A	0 / 5	0	0 / 5	0
	B	0 / 5		0 / 5	
	C	0 / 5		0 / 5	
	D	0 / 5		0 / 5	
100	A	0 / 5	0	0 / 5	0
	B	0 / 5		0 / 5	
	C	0 / 5		0 / 5	
	D	0 / 5		0 / 5	

Table 2 Observed abnormal response

Nominal concentration (mg/L)	Observed abnormal response			
	24 hours		48 hours	
	Immobilization	Other symptoms	Immobilization	Other symptoms
Control	-	-	-	-
100	-	-	-	-

- : Normal (No abnormal response)

Table 3 Condition of test solutions

Nominal concentration (mg/L)	Dissolved oxygen concentration (mg/L)		pH		Temperature (°C)	
	At the start	At the end	At the start	At the end	At the start	At the end
Control	8.8	8.9	7.8	7.7	20.3	20.4
100	8.8	8.9	7.7	7.7	20.3	20.4

Table 4 EC_{50} to *Daphnia magna*

Exposure duration	EC_{50} (mg/L)
24-hour	>100
48-hour	>100

Appendix 1

Chemical characteristics of dilution water

Chemical characteristics of dilution water (Sampling on January 31, 2017)

Parameter	Unit	Results	Determination limit
Total hardness (Ca, Mg)	mg/L	40	1
Suspended solid	mg/L	<1	1
pH	-	7.9 (23.5°C)	-
Total organic carbon	mg/L	<0.5	0.5
Chemical oxygen demand	mg/L	<1	1
Residual chlorine	mg/L	<0.02	0.02
Ammonium ion	mg/L	<0.1	0.1
Total cyanide	mg/L	<0.05	0.05
Alkalinity	mg/L	39	1
Electric conductivity	mS/m	15	0.1
Total mercury	mg/L	<0.0005	0.0005
Cadmium	mg/L	<0.001	0.001
Chromium (VI)	mg/L	<0.01	0.01
Lead	mg/L	<0.001	0.001
Arsenic	mg/L	<0.005	0.005
Iron	mg/L	<0.01	0.01
Copper	mg/L	<0.001	0.001
Cobalt	mg/L	<0.001	0.001
Manganese	mg/L	<0.005	0.005
Aluminum	mg/L	<0.02	0.02
Zinc	mg/L	<0.1	0.1
Nickel	mg/L	<0.001	0.001
Silver	mg/L	<0.0001	0.0001
1,2-dichloropropane	mg/L	<0.002	0.002
Chlorothalonil	mg/L	<0.001	0.001
Propyzamide	mg/L	<0.0008	0.0008
Chlornitrofen	mg/L	<0.0001	0.0001
Simazine	mg/L	<0.0003	0.0003
Thiobencarb	mg/L	<0.001	0.001
Diazinon	mg/L	<0.0005	0.0005
Isoxathion	mg/L	<0.0008	0.0008
Fenitrothion	mg/L	<0.0003	0.0003
EPN	mg/L	<0.0006	0.0006
Dichlorvos	mg/L	<0.001	0.001
Iprobenfos	mg/L	<0.0008	0.0008
PCB	mg/L	<0.0005	0.0005
Boron	mg/L	<0.1	0.1
Fluorine	mg/L	0.2	0.1
Sulfate ion	mg/L	15	0.5
Chloride ion	mg/L	12	0.2
Sodium	mg/L	14	0.2
Potassium	mg/L	3.4	0.2
Calcium	mg/L	11	0.1
Magnesium	mg/L	3.1	0.1

Appendix 2

Analytical method and measured concentration of test item

1. Pretreatment of test solution

The collected test solutions were used as the samples for high-performance liquid chromatography (HPLC) without treatment or after dilution with dechlorinated tap water.

2. Determination of test item

a) Method of determination

Determination of test item was conducted by absolute calibration curve method using one concentration of standard solutions.

The calibration curve was drawn by using four standard solutions of 1.00, 5.00, 10.0 and 20.0 mg/L which were prepared in the same way described in c) to confirm the effectiveness of this quantity method. As a result, the effectiveness was confirmed because the regression equation drawn from the relationship between the concentrations and the peak area on the each of chromatograms was confirmed as a straight line from origin. The drawn calibration curve and chromatograms which obtained by analysis of some samples for HPLC are shown in Appendix 3.

The determination limit of the test item in the test solution was the lowest concentration of the standard solution (1.00 mg/L) within the range of the calibration confirmed.

b) Analytical condition

Instrument	High-performance liquid chromatograph (Instrument No. LC-166)
Pump	LC-20AD (Shimadzu)
UV-VIS detector	SPD-20AV (Shimadzu)
Column oven	CTO-20A (Shimadzu)
Auto injector	SIL-20AC (Shimadzu)
System controller	SCL-10A$_{VP}$ (Shimadzu)
Degasser	DGU-20A$_3$ (Shimadzu)
Column	L-column2 ODS (150 mm × 2.1 mm I.D., particle size 5 μm, Chemicals Evaluation and Research Institute, Japan)
Column temp.	40°C
Eluent	A (50%) : Acetonitrile B (50%) : Ultra pure water/0.5 mol/L tetra-*n*-butylammonium phosphate solution (100/1 v/v)
Flow rate	0.2 mL/min
Wave length	215 nm
Injection volume	20 μL

c) Preparation of standard solution and calculation of test item concentration

The standard sample for analysis of the test item (50.1 mg) was precisely weighed by an electronic analytical balance and dissolved in ultra pure water fill up to 50 mL to obtain 1000 mg/L solution of the standard sample. The solution was diluted with dechlorinated tap water to prepare 10.0 mg/L standard solution

The concentration of the test item in each sample for HPLC analysis was determined on the basis of a comparison of the peak area on the chromatogram of the sample solution with that of a standard solution.

The standard sample for analysis of the test item (supplied by the sponsor)

Name	APFHx (C-1500N)
Purity	99.8%
Lot number	C150E57002
Storage condition	The standard sample was stored in a dark storage place at room temperature in a desiccator.
Appearance	White powder

The standard sample for analysis of the test item was treated with correcting by the purity of the test item.

4. Results of measurement

The results of the measured concentrations of the test item in the test solutions are shown below.

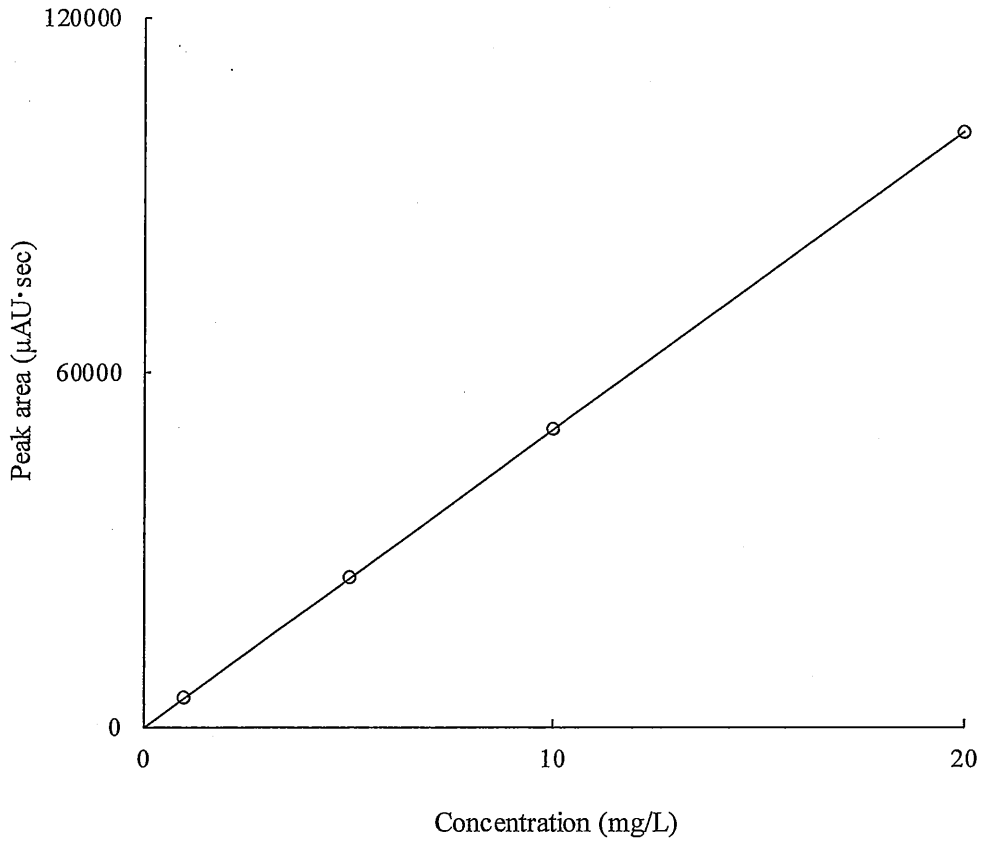
Appendix table 2-1 Measured concentrations of test item in test solutions

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus nominal concentration %)		
	At the start	At the end	Geometric mean
Control	n.d.	n.d.	
100	102 (102)	101 (101)	101 (101)

n.d. : <1.00 mg/L

Appendix 3

Calibration curve and chromatogram



y = 5031x

r = 1.00

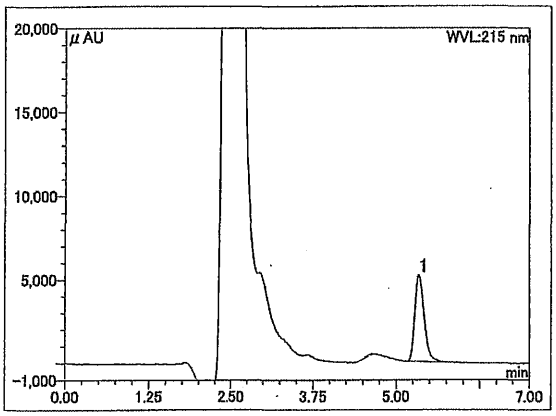
Concentration (mg/L)	Peak area (μAU·sec)
1.00	4917
5.00	25234
10.0	50276
20.0	100617

Appendix figure 3-1 Calibration curve of test item for analysis by HPLC.

Study No. 97726

Standard solution 10.0 mg/L

Operator:	Mika Ono
Operating date:	24/Apr/2017
Sample ID:	97726_170424_S2
Program:	97725_97726_iso
Vial No.:	1_1
Channel:	UV_VIS_1

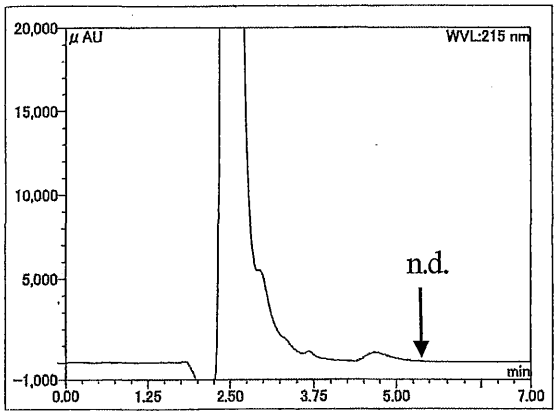


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	5.34	5157	49490	100.00
Total	–	–	49490	100.00

Study No. 97726

Control

Operator:	Mika Ono
Operating date:	24/Apr/2017
Sample ID:	97726_170424_H0hZ
Program:	97725_97726_iso
Vial No.:	1_2
Channel:	UV_VIS_1

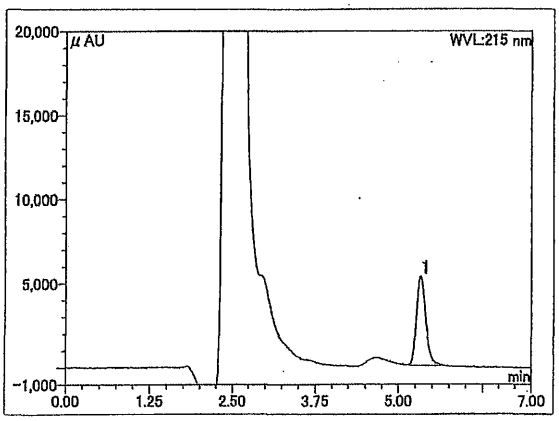


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
Total	–	–	0	0.00

Study No. 97726

100 mg/L exposure level

Operator:	Mika Ono
Operating date:	24/Apr/2017
Sample ID:	97726_170424_H0hA
Program:	97725_97726_iso
Vial No.:	1_3
Channel:	UV_VIS_1



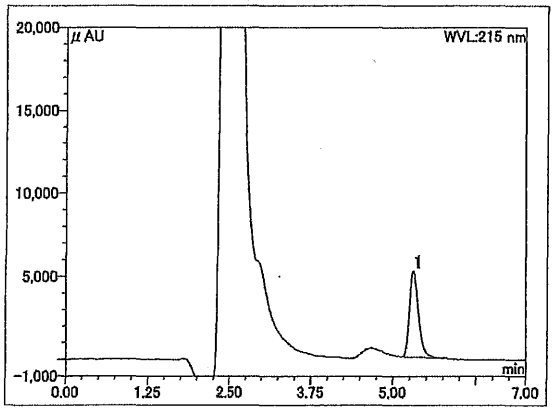
Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	5.33	5290	50455	100.00
Total	–	–	50455	100.00

Appendix figure 3-2 HPLC chromatograms at start of exposure.

Study No. 97726

Standard solution 10.0 mg/L

Operator:	Mika Ono
Operating date:	26/Apr/2017
Sample ID:	97726_170426_S2
Program:	97725_97726_iso
Vial No.:	1_1
Channel:	UV_VIS_1

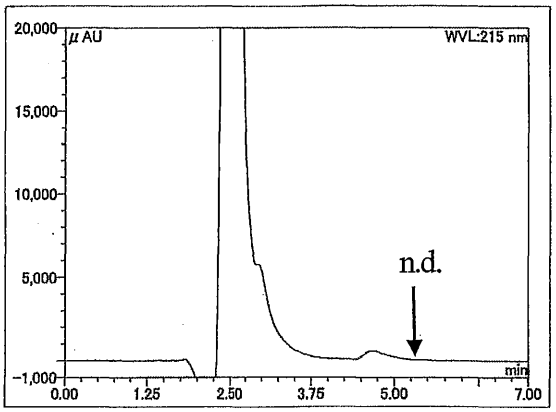


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	5.31	5195	49023	100.00
Total	-	-	49023	100.00

Study No. 97726

Control

Operator:	Mika Ono
Operating date:	26/Apr/2017
Sample ID:	97726_170426_H48hZ
Program:	97725_97726_iso
Vial No.:	1_2
Channel:	UV_VIS_1

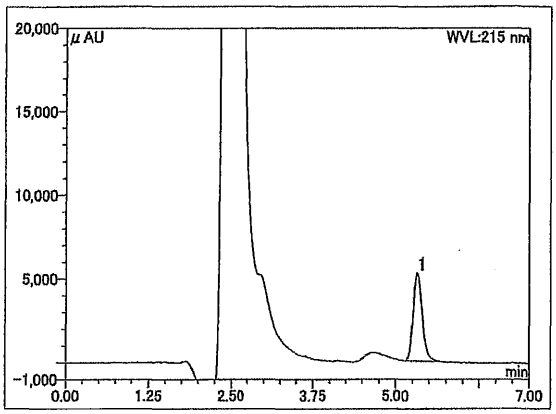


Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
Total	-	-	0	0.00

Study No. 97726

100 mg/L exposure level

Operator:	Mika Ono
Operating date:	26/Apr/2017
Sample ID:	97726_170426_H48hA
Program:	97725_97726_iso
Vial No.:	1_3
Channel:	UV_VIS_1



Peak No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	5.32	5246	49528	100.00
Total	-	-	49528	100.00

Appendix figure 3-3 HPLC chromatograms at end of exposure.

Additional data

Results of preliminary study

1. Solubility of test item in dilution water

It was confirmed that the solubility of the test item in dilution water was more than 100 mg/L for visual observation.

2. Preliminary studies of effect on test organism

Type of test	Static regime
Number of test organisms	10 test organisms/test level (5 test organisms/test vessel)
Preparation of test solution	The test sample was dissolved in dilution water by mixing and stirring to prepare the test solution.
Analytical chemistry	The test item concentrations in the test solution were measured.

<Result of effect on test organisms>

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Observed symptoms	Immobility (%)	Observed symptoms
100	0	-	0	-

- shows that no other abnormal response was observed.

<Measured concentration of test item in test solution>

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus that of nominal concentration %)	
	At the start of exposure	At the end of exposure
100	103 (103)	101 (101)

3. Condition of definitive study

Test level	100 mg/L and a control
Type of test	Static regime