CERI

Receipt number	662-06-E-4227
Study number	94227

FINAL REPORT

A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*

July 26, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*

Study number 94227

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94227, issued on July 26, 2007)

Date September 10, 2009

Study Director

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*

Study number 94227

The study described in this report was conducted in compliance with the following GLP principles:

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
(2) "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 July 26, 2007

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*

Study number 94227

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 and audit 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 / audit	Date of inspection / audit	Date of report to Study Director and Test Facility Management
Study plan draft	June 18, 2007	June 18, 2007
Study plan	June 18, 2007	June 18, 2007
Measurement of solubility	June 20, 2007	June 22, 2007
	June 21, 2007	June 22, 2007
Start of the exposure and after the exposure	June 19, 2007	June 22, 2007
	June 20, 2007	June 22, 2007
	June 22, 2007	June 22, 2007
Raw data and final report draft	July 21, 2007	July 24, 2007
Final report	July 26, 2007	July 26, 2007

Date July 26, 2007

Head of Quality Assurance Unit Signed in original

CONTENTS

Page

Title 1

Sponsor 1

Test facility 1

Objective 1

Test method 1

Applied GLP 1

Dates 2

Storage of test item, raw data, etc. 2

Personnel 3

Approval of final report 3

SUMMARY 4

1. Test item 6
2. Test sample 7
3. Test materials and methods 8
4. Results and discussion 12
5. Factors that affected reliability of test results 14
6. Content of deviation from protocol 14

Tables

Table 1	Immobility
Table 2	Observed abnormal response
Table 3-1	Dissolved oxygen concentration of test solutions
Table 3-2	pH of test solutions
Table 3-3	Temperature of test solutions
Table 4	EC_{50} to *Daphnia magna*
Appendix 1	Chemical characteristics of dilution water
Appendix 2	Analytical method and measured concentration of test item and 13F-EtOH (hydrolyzed product)
Appendix 3	Calibration curve and chromatogram
Appendix 4	Solubility of test item in dilution water
Additional data	Results of preliminary studies

Title	A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*
Sponsor	DAIKIN INDUSTRIES, LTD. 1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585, Japan
Test facility	Kurume Laboratory Chemicals Evaluation and Research Institute, Japan 3-2-7, Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan
Objective	The objective of this study is to estimate the acute toxicity of 13F-SFMA to *Daphnia* sp.
Test method	This study was performed according to the following test methods and guidance document. (1) *Daphnia* sp., Acute Immobilization Test stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003; No. 1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No. 2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121002, Environmental Policy Bureau, Ministry of the Environment) (2) OECD Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, 202 "Daphnia sp., Acute Immobilisation Test (Guideline 202, April 13, 2004)" (3) OECD Guidance Document No. 23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures" (September 2000)
Applied GLP	This study was conducted in compliance with the following GLP principles: (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment) (2) "OECD Principles of Good Laboratory Practice" (November 26, 1997, ENV/MC/CHEM (98)17).

Dates

Study initiation date	June 18, 2007
Experimental starting date	June 20, 2007
Solubility study starting date	June 20, 2007
Bioassay starting date	June 20, 2007
Experimental completion date	June 22, 2007
Solubility study completion date	June 21, 2007
Bioassay completion date	June 22, 2007
Study completion date	July 26, 2007

Storage of test item, raw data, etc.

(1) Test item

The test sample*1 will be sealed in a storage vessel and stored in archives in this laboratory for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". If it is not stable for the storage period, it is stored as long while it is kept stable. Treatment of the sample after the storage period will be discussed with sponsor.

*1 It will be stored as the common sample for storage of these studies (Study Nos. 94226, 94227 and 94228).

(2) Raw data and materials

Raw data, the study plan, documents concerning the study presented by the sponsor, the final report and necessary materials will be stored in archives in this laboratory for ten years after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period will be discussed with sponsor.

Personnel

Study Director :

Study personal

Biology :

Analytical chemistry :

Approval of final report

Study Director	Date	July 26, 2007
	Signature	Signed in original

SUMMARY

Title

A 48-hour Acute Immobilization Study of 13F-SFMA with *Daphnia magna*

Test conditions

(1) Test item	13F-SFMA
(2) Test organism	*Daphnia magna*
(3) Exposure duration	48 hours
(4) Test concentration	Middle layer of suspended solution (nominal concentration: 100 mg/L), and control
(5) Number of organisms	Twenty daphnids / test level (five daphnids / test vessel)
(6) Dilution water	Dechlorinated tap water
(7) Type of test	Semi-static regime (renewal at 24 hours after) with closed system
(8) Preparation of test solution	The test sample and dilution water were mixed to produce 100 mg/L (nominal concentration), and they were stirred under closed system for approx. 24 hours. After settlement for 1 hour, test solution was prepared by taking out from the middle layer.
(9) Replicate	Four replicates / test level
(10) Volume of test solution	Approx. 1000 mL / test level (approx. 250 mL / test vessel)
(11) Temperature of test solutions	20±1°C
(12) Irradiation condition	Artificial light of white fluorescent lamp, 16-hour light / 8-hour dark
(13) Feeding	No feeding
(14) Aeration	No aeration

(15) Analysis of concentration of test item and hydrolyzed product in test solution

GC analysis (at the start of the exposure, before and after the renewal, and the end of the exposure)

Results

(1) Solubility in dilution water (20±1°C)	0.0587 mg/L
(2) Concentration of test item in test solution (Percentage of concentration at preparation)	
At the start of the exposure and after the renewal	0.0508 and 0.0426 mg/L
Before the renewal and at the end of the exposure	0.0299 and 0.0308 mg/L (59.0 and 72.3%)
(3) Concentration of 13F-EtOH in test solution	
At the start of the exposure and after the renewal	<0.00592 mg/L (below the determination limit)
Before the renewal and at the end of the exposure	0.00606 and 0.00647 mg/L
(4) 48-hour EC_{50} (Median Effective Concentration)	> 0.0376 mg/L

[The concentration shown in (4) is based on a geometric mean of the measured concentrations.]

Conclusion

This study was conducted as a limit test at the concentration around solubility of the test item in dilution water to confirm the effect on the test organisms. It was concluded that the test item has no acute toxicity to the test organisms at the concentration around water solubility, since the measured concentrations of the test solutions at the preparation were around the solubility in dilution water and no effect on the test organisms was observed under the test condition.

1. Test item

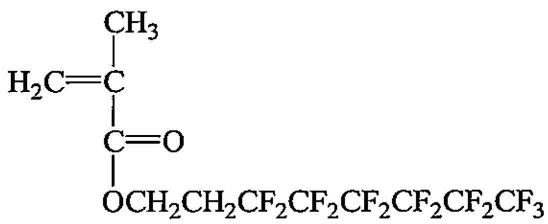
In this report, 13F-SFMA has the following name etc.

1.1 Chemical name[*2]

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl metacrylate

1.2 Chemical structure etc.[*2]

Structural formula



Molecular formula	$C_{12}H_9F_{13}O_2$
Molecular weight	432.18
CAS Number	2144-53-8

*2 Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number*2

Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	6Y002

2.2 Purity*2

Test item	99.8%	
Impurity	Unknown constituent component	0.2%

2.3 Confirmation of test item

It was confirmed that infrared (IR) spectrum of the test item provided by the sponsor coincided with IR spectrum analyzed in this laboratory.

2.4 Physicochemical properties*2

Appearance at normal temperature	Colorless and clear liquid	
Boiling point	92°C (8 mmHg)	
Density	1.496 g/cm^3 (25°C)	
Solubility	Water	Insoluble
	Dimethylsulfoxide	Soluble (fully miscible)
	Acetone	Soluble (fully miscible)

*2 Information supplied by the sponsor

2.5 Storage condition and confirmation of stability at storage condition

Storage condition	Dark storage place at room temperature
Confirmation of stability	The stability of the test item during the test period was confirmed by no alteration in the IR spectra of the test item before the experimental start and after the experimental completion.

3. Test materials and methods

3.1 Test organism

(1) Species

Daphnia magna (Clone A)

(2) Reason for selection of species

Species recommended in the test guidelines

(3) Source

Young daphnids produced by parents which were cultured in the Kurume Laboratory were used. Daphnids [*Daphnia magna* (Clone A)] originally came from the University of Sheffield (Address: Sheffield S10 2UQ, United Kingdom). The parents to obtain young daphnids were bred in the same quality of water (dechlorinated tap water), water temperature (20±1°C), photoperiod (16-hour light / 8-hour dark) as used in the test. Parents used for the test were same lot and bred for more than 14 days, and their age and survival rate were 21-day old and 100%, respectively. *Chlorella vulgaris* of 0.1 to 0.2 mgC/day per *daphnia* was fed to the parents once a day. A 48-hour acute immobilization test of $K_2Cr_2O_7$ (Reagent grade, Wako Pure Chemical Industries, Ltd.) with the test organisms was conducted (on April 9 to 11, 2007) to confirm the reproducibility of the test conditions. The 48-hour EC_{50} of $K_2Cr_2O_7$ was 0.270 mg/L. This value was within the normal range in this laboratory (mean ± 2S.D.: 0.122 to 0.350 mg/L) [mean ± S.D.: 0.236± 0.057 mg/L (n=58)].

(4) Selection of young daphnids

Less than 24-hour old daphnids were used for the test.

(5) Allocation to the test groups

Test organisms were placed at random to each test vessel.

3.2 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.

3.3 Test apparatus and equipment

(1) Test apparatus

Test vessel : Petri dish (diameter: 8.0 cm, depth: 5.0 cm)

The test vessels were covered and closed with glass lid in order to prevent dust, and volatilization of the test solution.

(2) Test equipment

Water bath : Plastic tank
Warming / cooling unit (Type HCA250, Sato craft)

3.4 Test conditions

(1) Conditions of exposure

(a) Type of test

The test organisms were exposed to the test solution containing the test item.

The test was conducted as semi-static regime of whole test solution replacement after 24 hours with closed system.

(b) Exposure duration

48 hours

(c) Test concentration

Based on the results of the preliminary studies, it was expected that the test solution at around the solubility in dilution water would have no immobility for the test organism. Therefore, the definitive study was conducted as the limit test with suspended solution which was prepared by taking out from the middle layer of 24-hour mixed solution (nominal concentration: 100 mg/L). The results of the preliminary studies are shown in Additional data.

(d) Control

The dilution water without the test item, which was treated in the same stirring manner as the test solution, was used as the control.

(e) Replicates

Four replicates/test level

(f)Number of organism

Twenty daphnids / test level (five daphnids / test vessel)

(g) Volume of test solution

Approx. 1000 mL / test level (Approx. 250 mL / test vessel)

(2) Conditions of test environment

(a) Water temperature

20±1°C

(b) Dissolved oxygen concentration

The study was performed in the condition where dissolved oxygen concentration was more than 3 mg/L. No aeration was used for the test during the exposure.

(c) pH

The test was performed without adjusting pH.

(d) Irradiation condition

Artificial light of white fluorescent lamp, 16-hour light/8-hour dark

(e) Feeding

Test organisms were not fed during the exposure.

3.5 Preparation of test solution

No correction with purity was done for the preparation of the test concentration. The test sample was employed in terms of volume using the density [1.496 g/cm^3 (25°C)] for the preparation of test solution.

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for approx. 24 hours under approx. 20°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. The prepared test solution was immediately divided into each test vessel and covered with glass lid not to produce head space.

3.6 Observation and measurements

(1) Observation of test organisms

Immobility and symptom were observed at 24 and 48 hours after the exposure. Daphnids were considered immobile if they were not able to swim within 15 seconds after gentle agitation of the test vessel.

(2) Appearance of test solution

Appearance of the test solutions was observed at the start and before the renewal (after 24 hours).

(3) Condition of test solutions

Dissolved oxygen concentration, pH and temperature of the test solutions were measured at the start of the exposure, before and after the renewal, and at the end of the exposure (twice of a set of preparation and 24 hours after). At the start of the exposure, another solution sampled from the container for preparation was used for the measurement. At 24 hours after the preparation, the measurement was carried out for one test vessel in each level. The dissolved oxygen concentration measurements were carried out with an oxygen meter (YSI Model 58, YSI Incorporated.). The pH measurements were carried out with a pH meter (Model HM-21P, DKK-TOA). The temperature measurements were carried out with a calibrated red alcohol thermometer of glass stick type.

(4) Concentration of test item in test solution

The concentration of the test item in the test solutions was measured at the start of the exposure, before and after the renewal, and the end of the exposure (twice of a set of preparation and 24 hours after). Since the test item was hydrolyzed and produced 2-(perfluorohexyl)ethanol (abbreviation: 13F-EtOH which is the test item of study number 94232 to 94234), the concentration of 13F-EtOH was also measured. At the start of the exposure, another solution sampled from the container for preparation was used for analysis. At 24 hours after the preparation, the test solution for analysis was taken out with equal volume from the middle layer of the test solution in test vessels in each test level, and mixed. The concentrations of the test item and 13F-EtOH were analyzed by gas chromatography (GC). Analytical method and measured concentration of test item 13F-EtOH are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(5) Solubility of test item in dilution water

Since the solubility of the test item was expected less than 100 mg/L, it was measured concurrently with the definitive study. The detail and the result of the measurement of the solubility are shown in Appendix 4.

3.7 Calculating method of EC_{50} [*3]

The EC_{50} value was estimated as "> test concentration" since no less than 50% of immobility was not observed in the present exposure level.

The results of the test were estimated based on a geometric mean of the measured concentrations.

*3 EC_{50} (Median Effective Concentration) is the concentration at which causes 50% immobility of tested population during exposure.

3.8 Validity of the test

(1) The immobilization rate should not exceed 10% in control group during exposure.

(2) 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.

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

3.9 Treatment of numerical values

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

(JIS ; Japanese Industrial Standards)

4. Results and discussion

4.1 Immobility

No immobility of the test organism was observed in the exposure level during exposure. Immobility at 24 and 48 hours are shown in Table 1. In the control, no trapping daphnids at the surface was observed, and immobility during the exposure was 0%, which meets the criterion for the validity of the test (i.e. not more than 10%).

4.2 Observed abnormal response

There was no abnormal response in the control.

The following results of observation were based on the comparison with the control organisms. No abnormal responses were obtained in the test level during exposure. The result of the observation during exposure is shown in Table 2.

4.3 Observation and measurement of test solution

(1) Appearance of test solution

At the start of the exposure, the test solution was colorless and clear. The appearance kept until before the renewal.

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature of the test solution during the exposure were 8.2 to 8.4 mg/L, 7.7 to 7.8, and 19.8 to 20.0°C, respectively. Conditions of the test solutions are shown in Table 3-1, 3-2 and 3-3. The measured values of dissolved oxygen concentration met the criterion for the validity of the test (more than 3 mg/L at the end of exposure).

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.0508 and 0.0426 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.0299 and 0.0308 mg/L which were 59.0 and 72.3% of the concentration at the preparation. The measured concentrations of 13F-EtOH in the test solution were <0.00592 mg/L (below the determination limit) at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.00606 and 0.00647 mg/L. The results of the measured concentrations of the test item and 13F-EtOH are shown in Appendix 2.

4.4 EC_{50}

Both the 24-hour and 48-hour EC_{50}s of 13F-SFMA to *Daphnia magna* were >0.0376 mg/L (based on a geometric mean of the measured concentrations). The EC_{50}s at each observation time are shown in Table 4.

4.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at the concentration around the solubility of the test item in dilution water. Although one of the measured concentrations of the test item during the exposure was low (0.0426 mg/L) compared with the solubility (0.0587 mg/L) measured concurrently with the definitive study, it was thought that the concentrations of the test item in the test solution at the preparation were around the solubility because the values measured in the solubility study fluctuated and the concentration of 0.0402 mg/L was included in them. Although the measured concentrations at 24 hours after the preparation decreased, it was supposed that the study was appropriate as a test around the solubility of the test item in the dilution water because semi-static system, i.e. renewal of the test solution at 24 hours after the exposure, was chosen to maintain the test concentration. Since no adverse effect was observed under the condition in the definitive study, it was concluded that the test item had no adverse acute effect on the test organisms at the concentration around the solubility in dilution water. On the other hand, the concentration of 13F-EtOH which was hydrolyzed product of the test item tended to increase in the test solution, and the measured concentrations of it ranged from <0.00592 (below the determination limit) to 0.00647 mg/L. However, it was concluded that the hydrolyzed product had no adverse effect on the test organisms due to the result of the study.

5. Factors that affected reliability of test results

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

6. Content of deviation from protocol

None.

Table 1 Immobility

Measured Concentration*4 (mg/L)		Immobility (%)			
		24 hour		48 hour	
		Replicate	Test level	Replicate	Test level
Control	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	
0.376	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	

*4: geometric mean of measured concentration

(The followings are expressed as measured concentration)

Table 2 Observed abnormal response

Measured concentration (mg/L)	Observed abnormal response	
	24 hours	48 hours
Control	-	-
0.376	-	-

- : No abnormal response

Table 3-1 Dissolved oxygen concentration of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	8.2	8.4	8.3	8.3
0.0376	8.3	8.3	8.4	8.3

Unit : mg/L

Table 3-2 pH of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	7.7	7.7	7.7	7.7
0.0376	7.8	7.7	7.7	7.7

Table 3-3 Temperature of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	19.8	20.0	20.0	20.0
0.0376	20.0	20.0	20.0	20.0

Unit : °C

Table 4 EC_{50} to *Daphnia magna*

Exposure duration	EC_{50} (mg/L)	95% confidence interval (mg/L) (Slope of the dose-response curve)	Statistical procedure used for determination of EC_{50}
24-hour	>0.0376	- (-)	-
48-hour	>0.0376	- (-)	-

- : Not obtained

Appendix 1

Chemical characteristics of dilution water

Chemical charasteristics of dilution water (Sampling on January 9, 2007)

Parameter	Unit	Results	Lower limit of determination
Total hardness (as $CaCO_3$)	mg/L	41.9	0.1
Suspended solid	mg/L	< 1	1
pH	—	7.9 (22°C)	—
Total organic carbon	mg/L	0.2	0.1
Chemical oxygen demand	mg/L	0.7	0.5
Residual chlorine	mg/L	< 0.02	0.02
Ammonium ion	mg/L	0.01	0.01
Total cyan	mg/L	< 0.01	0.01
Alkalinity	mg/L	35	1
Electric conductivity	mS/m	18.3	—
Organic phosphorous	mg/L	< 0.1	0.1
Alkylmercury	mg/L	< 0.0005	0.0005
Mercury	mg/L	< 0.0005	0.0005
Cadmium	mg/L	< 0.001	0.001
Chromium (VI)	mg/L	< 0.02	0.02
Lead	mg/L	< 0.005	0.005
Arsenic	mg/L	< 0.001	0.001
Boron	mg/L	0.08	0.02
Fluorine	mg/L	< 0.1	0.1
Iron	mg/L	< 0.01	0.01
Copper	mg/L	< 0.005	0.005
Cobalt	mg/L	< 0.001	0.001
Manganese	mg/L	< 0.01	0.01
Zinc	mg/L	< 0.01	0.01
Aluminum	mg/L	0.033	0.001
Nickel	mg/L	< 0.001	0.001
Silver	mg/L	< 0.0001	0.0001
Sulfate ion	mg/L	3.9	0.1
Chloride ion	mg/L	16	1
Sodium	mg/L	14.3	0.01
Potassium	mg/L	3.7	0.01
Calcium	mg/L	11.5	0.01
Magnesium	mg/L	3.2	0.01
1,2-dichloropropane	mg/L	< 0.0001	0.0001
Chlorothalonil	mg/L	< 0.0001	0.0001
Propyzamide	mg/L	< 0.0001	0.0001
Chlornitrofen	mg/L	< 0.0001	0.0001
Simazine	mg/L	< 0.001	0.001
Thiobencarb	mg/L	< 0.0001	0.0001
Diazinon	mg/L	< 0.0001	0.0001
Isoxathion	mg/L	< 0.0001	0.0001
Fenitrothion	mg/L	< 0.0001	0.0001
EPN	mg/L	< 0.0001	0.0001
Dichlorvos	mg/L	< 0.0001	0.0001
Iprobenfos	mg/L	< 0.0001	0.0001
PCB	mg/L	< 0.0005	0.0005

Appendix 2

Analytical method and measured concentration of test item
and 13F-EtOH (hydrolyzed product)

1. Analysis of test solution

By the result of preliminary study, it was expected that the test item was hydrolyzed into 13F-EtOH during the exposure. Therefore, the concentrations of the test item and 13F-EtOH were measured.

2. Pretreatment of test solution

The test solutions sampled were pretreated according to the following flow scheme to prepare the sample for analysis.

Flow scheme

Test solution 100 mL

←Sodium chloride 30 g (electronic even balance)
←Ethyl acetate 30 mL (graduated cylinder)
・Shaking (10 min)

Water layer | Ethyl acetate layer

・Transferring erthyl acetate layer to the eggplant flask with a beaker
・Concentration to approx. 1 mL (rotary evaporator, approx. 40°C)
・Filling up to 2 mL (ethyl acetate, volumetric flask)

Sample for analysis

3. Method of analysis

The pretreated samples for analysis were quantitatively analyzed by gas chromatography (GC) under the following conditions to determine the concentrations of the test item and 13F-EtOH (hydrolyzed product). The concentrations of the test item and 13F-EtOH in each sample for GC analysis were 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 samples that the concentration of the test item and 13F-EtOH exceeded the range of the calibration curve were diluted to fall within the range and analyzed. Some chromatograms obtained are shown in Appendix 3.

Analytical conditions

Instrument	Gas chromatograph
	Hewlett Packard HP 6890 Series GC System
Auto injector	Hewlett Packard HP6890 Series
Detector	Flame ionization detector (FID)
Column	DB-WAX film thickness 0.50 μm
	(Agilent Technologies)
	30 m × 0.32 mm I.D. Fused silica
Column temp.	40°C (5 min) $\xrightarrow{①}$ 150°C (0 min) $\xrightarrow{②}$ 240°C (2 min)
Temp. rate	①15°C/min ②50°C/min
Injection temp.	200°C
Carrier gas	Helium
Column flow	1.8 mL/min
Hydrogen	40.0 mL/min
Air	400 mL/min
Injection volume	2 μL
Inlet mode	Splitless
Purge flow	20.0 mL/min
Purge time	0.50 min
Detector	
Temp.	240°C
Sensitivity	Range 2^0

4. Preparation of standard solution

The standard solutions to determine the concentrations of the test item and 13F-EtOH in the sample for analysis were prepared as follows. The standard solution was each prepared with correcting by the purity (99.8%) of the test item and the purity (99.8%) of 13F-EtOH.

The test sample of 100.2 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of the test item. The reference standard for 13F-EtOH component analysis (the test sample of study number 94232-94234) of 100.2 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of 13F-EtOH. The test item solution was diluted with ethyl acetate to prepare 25.0 mg/L (as the concentrations of the test item and 13F-EtOH) of test item and 13F-EtOH solution, after 13F-EtOH solution was added. The solution was diluted with ethyl acetate to prepare 2.50 mg/L (as each concentrations of the test item and 13F-EtOH) of standard solution.

5. Calibration curve

The standard solutions of 0.250, 1.25, 2.50 and 5.00 mg/L (as the concentrations of the test item and 13F-EtOH) were prepared by the same procedure as described in section 4. These solutions were analyzed according to the quantitative analytical conditions described in section 3. The calibration curves were drawn from the relationship between the concentrations of standard solution (the test item and 13F-EtOH) and the peak area on the chromatogram, and the determination was confirmed. The calibration curve is shown in Appendix 3. The determination limit of the test item was the lowest concentration of the standard solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of the test item in the test solution was 0.00592 mg/L in consideration of pretreatment. The determination limit of 13F-EtOH was the lowest concentration of the standard solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of 13F-EtOH in the test solution was 0.00592 mg/L in consideration of pretreatment.

6. Recovery test and blank test

6.1 Method

The recovery test was conducted by adding the test item solution (prepared with acetone) to dechlorinated tap water according to pretreatment of test solution described in section 2. Similarly, the recovery test was conducted by adding 13F-EtOH solution (prepared with acetone) to dechlorinated tap water. The blank test was also conducted using dechlorinated tap water (added acetone) without the test item and 13F-EtOH in the same way as the recovery test. The recovery test and the blank test were performed in duplicate.

Amount of the test item added	5.00 μg
Amount of 13F-EtOH added	5.00 μg

6.2 Result

As a result of analysis by the method of section 6.1, no peak of the test item and 13F-EtOH appeared in the chromatogram of the blank test. Two individual recovery rates and their average on the analytical procedure are shown below. The averages of recovery rate were used as correction factor, for the determination of the test item and 13F-EtOH concentrations in the test solutions.

Recovery rate of the test item for pretreatment

82.1 %, 86.8 % average 84.4 %

Recovery rate of 13F-EtOH for pretreatment

82.9 %, 86.0 % average 84.5%

7. Results of measurement

The results of the measured concentrations of the test item and 13F-EtOH 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 that at each preparation %)				
	At the start	24 hours		At the end	Geometric mean
		Before the renewal	After the renewal		
Control	n.d.	n.d.	n.d.	n.d.	
100	0.0508	0.0299 (59.0)	0.0426	0.0308 (72.3)	0.0376

n.d. : <0.00592 mg/L

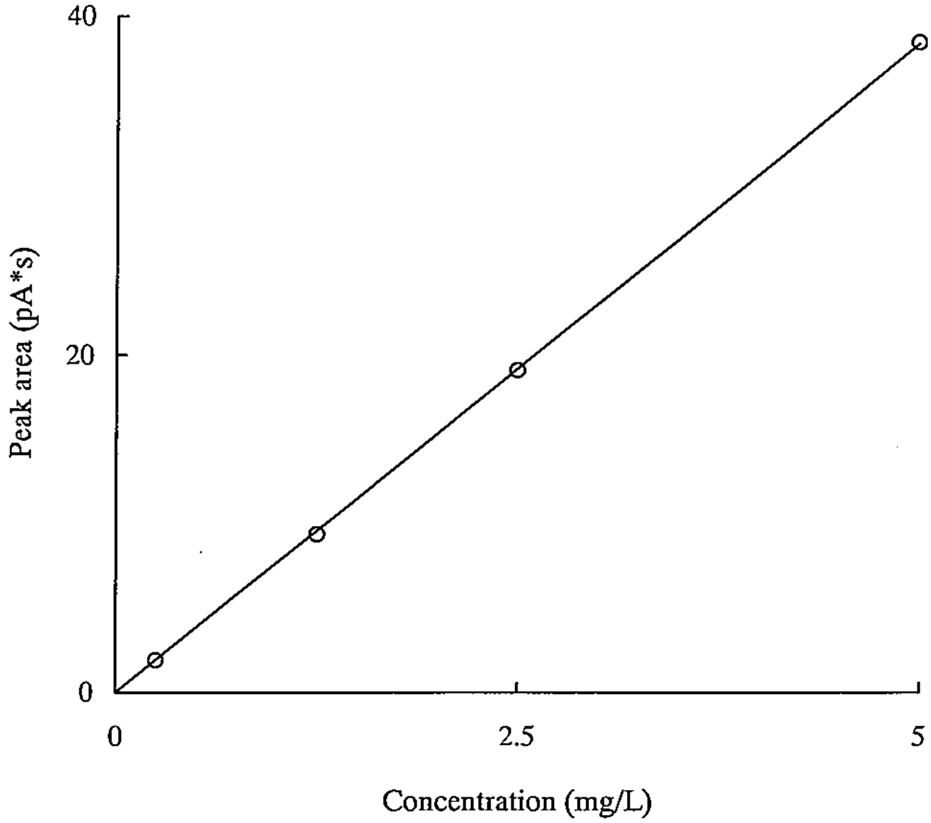
Appendix table 2-2 Measured concentrations of 13F-EtOH in test solutions

Nominal concentration (mg/L)	Measured concentration (mg/L)			
	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	n.d.	n.d.	n.d.	n.d.
100	n.d.	0.00606	n.d.	0.00647

n.d. : <0.00592 mg/L

Appendix 3

Calibration curve and chromatogram



y = 7.67x
r = 1.00

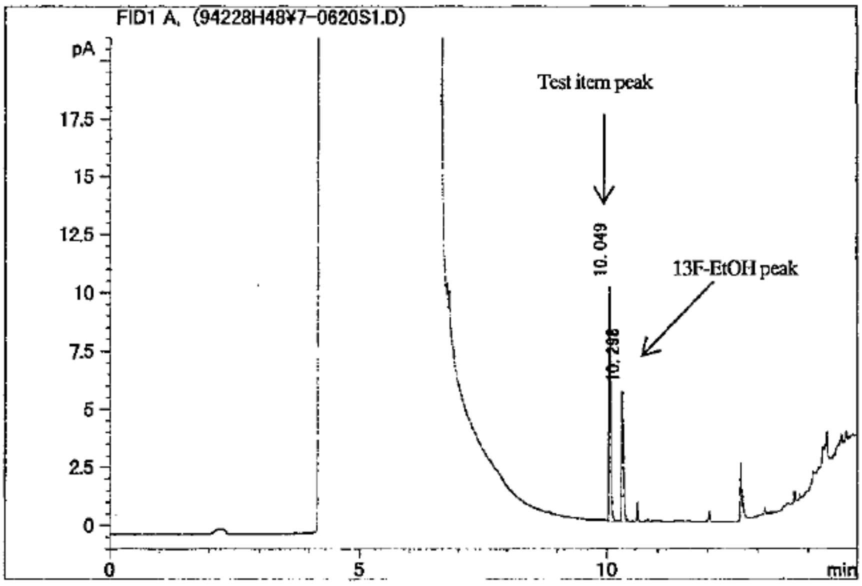
Concentration (mg/L)	Peak area (pA*s)
0.250	1.899
1.25	9.380
2.50	19.076
5.00	38.431

Appendix figure 3-1 Calibration curve of 13F-SFMA for analysis by GC.

Standard solution 2.50 mg/L

Study No. 94227

測定オペレータ　　:
メソッド名　　　: C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94228H48¥7-0620S1.D
サンプル名　　　: std 2.50mg/L



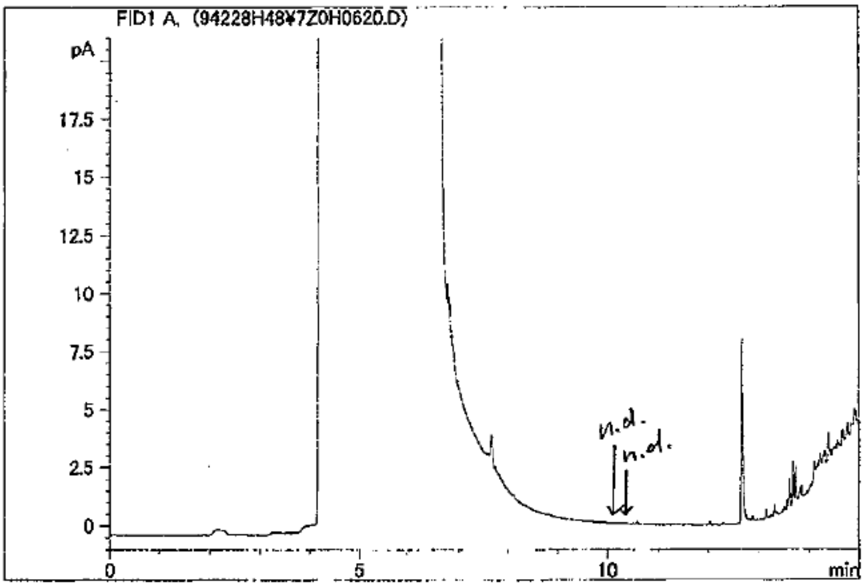
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.163	10.072	10.049
2	12.324	5.593	10.298

Appendix figure 3-2-1　GC chromatogram at start of exposure.

Control

Study No. 94227

測定オペレータ　：
メソッド名　：C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名：C:¥HPCHEM¥1¥DATA¥94228H48¥7Z0H0620.D
サンプル名　：Cont. (*50) H0h



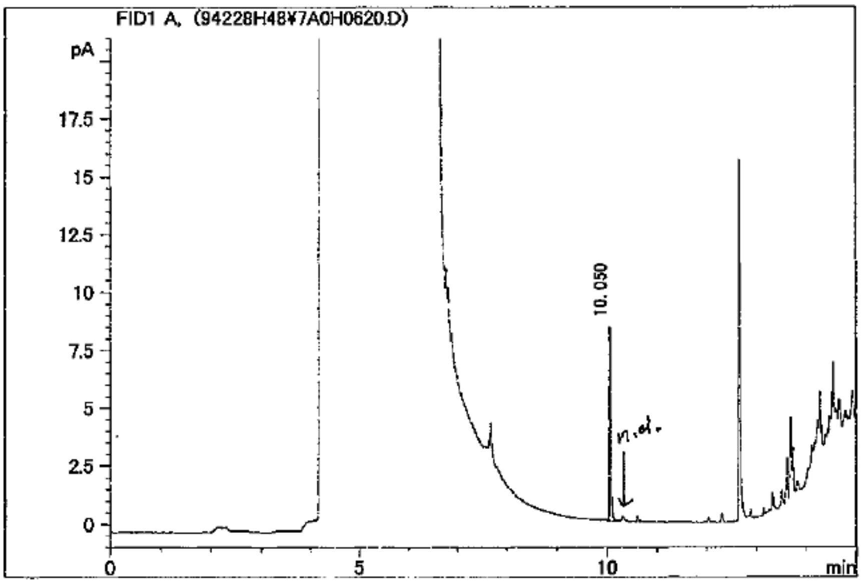
#	メインピーク面積	メインピーク高さ	実測 RT

Appendix figure 3-2-2　GC chromatogram at start of exposure.

100 mg/L (Nominal concentration)

Study No. 94227

測定オペレータ　　:
メソッド名　　　: C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94228H48¥7A0H0620.D
サンプル名　　　: 100(*50)H0h



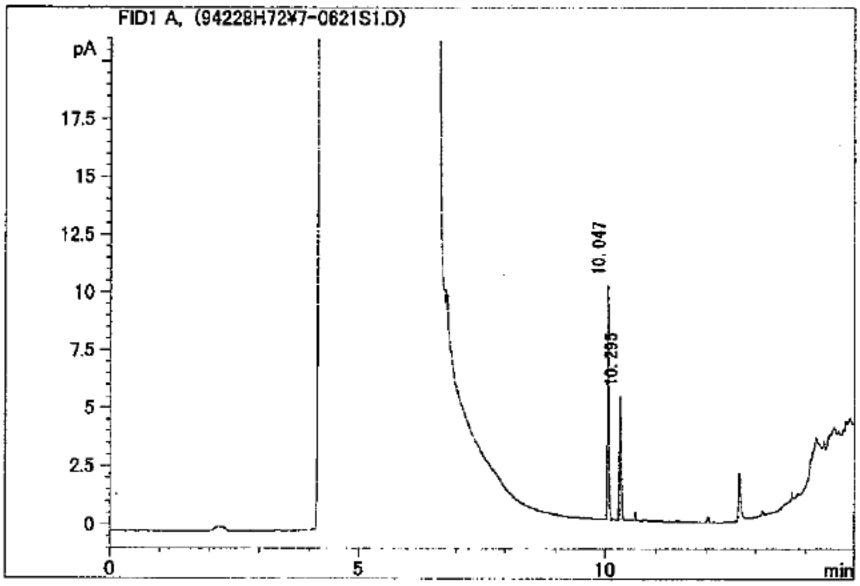
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	16.417	8.396	10.050

Appendix figure 3-2-3　GC chromatogram at start of exposure.

Standard solution 2.50 mg/L

Study No. 94227

測定オペレータ　　:
メソッド名　　　　: C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94228H72¥7-0621S1.D
サンプル名　　　　: std 2.50mg/L



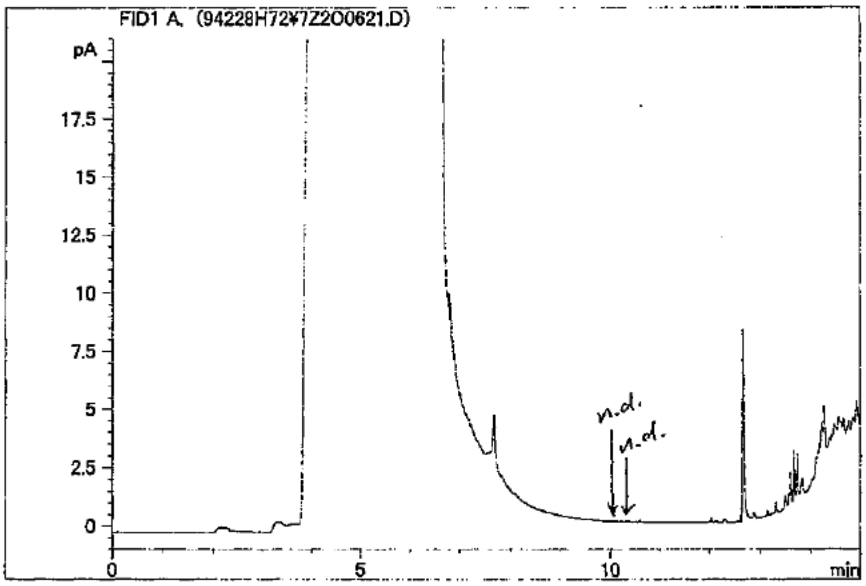
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.099	10.163	10.047
2	12.214	5.349	10.295

Appendix figure 3-3-1　GC chromatogram before renewal at 24 hours.

Control

Study No. 94227

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94228H72¥7Z200621.D
サンプル名 : Cont. (*50)H24old



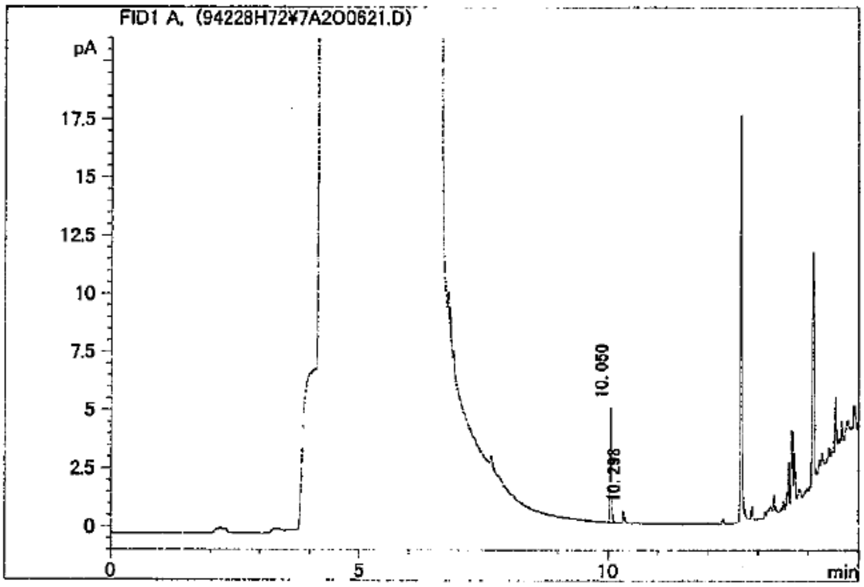
#	メインピーク面積	メインピーク高さ	実測 RT

Appendix figure 3-3-2 GC chromatogram before renewal at 24 hours.

100 mg/L (Nominal concentration)

Study No. 94227

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94226.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94228H72¥7A200621.D
サンプル名 : 100(*50)H24old



#	メインピーク面積	メインピーク高さ	実測 RT
1	9.647	4.960	10.050
2	1.251	0.527	10.298

Appendix figure 3-3-3 GC chromatogram before renewal at 24 hours.

Appendix 4

Solubility of test item in dilution water

1. Title

Solubility of test item in dilution water

2. Objective

The objective of this study is to estimate the solubility of the test item to dilution water.

3. Outline

Test item mixed with dilution water was stirred for 24 hours under the test temperature. After clearing insoluble matter, solubility was analyzed.

4. Performance of test

4.1 Test equipments and instruments

Water bath :	Plastic tank
	Warming/cooling unit (Type HCA250, Sato craft Ltd.)
Mixing apparatus :	Magnetic stirrer
Vessel :	Devised glass container
	(Interior volume : approx. 600 mL)

4.2 Test conditions

(1) Test temperature : 20±1°C

(2) Number of measurement : Once (after the mixture was stirred for 24 hours)

(3) Dilution water : Dechlorinated tap water

(4) Repitition : n=3 (Sample-1, Sample-2 and Sample-3)

4.3 Test procedures

(1) Test sample and dilution water were mixed in a devised glass container to prepare approx. 100 mg/L* solution and sealed without headspace.

* The additive amount (40.1 μL) was caluculated from the density of the test otem (1.496 g/cm^3).

(2) The test solution was stirred slowly with a magnetic stirrer under the test temperature in a water bath.

(3) After the solution was stirred for 24 hours, the flask was settled in a water bath for approx. 1 hour.

(4) After settling, the samples were quantitatively analyzed to measure the concentration of the test item and 13F-EtOH (hydrolyzed product).

4.4 Analysis of test solution

(1) Pretreatment of test solution

The middle layer of the test solutions were collected carefully from sampling spout of the devised glass container by syringe. The collected solution was pretreated according to the flow scheme described in Appendix 2 (2. Pretreatment of test solution).

(2) Method for analysis

See Appendix 2 3. Method of analysis.

4.5 Preparation of standard solution

See Appendix 2 4. Preparation of standard solution.

4.6 Calibration curve

See Appendix 2 5. Calibration curve.

4.7 Recovery test and blank test

See Appendix 2 6. Recovery test and blank test

5. Results

Measured solubility of the test item in dilution water was 0.0587 mg/L. In addition, the measured concentration of 13F-EtOH was below determination limit (0.00592 mg/L). The results of analyses are shown below.

Appendix table 4-1 Value measured after stirring for 24 hours (test item)

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-1	0.0452	0.0587
Sample-2	0.0906	
Sample-3	0.0402	

Appendix table 4-2 Value measured after stirring for 24 hours (13F-EtOH)

Sample name	Measured value (mg/L)	Arithmetic mean (mg/L)
Sample-1	n.d.	n.d.
Sample-2	n.d.	
Sample-3	n.d.	

n.d. : <0.00592 mg/L

Additional data

Results of preliminary studies

1. Solubility of test item in dilution water

It was expected that the solubility of the test item in dilution water was below 100 mg/L, therefore, the measurement of the solubility of the test item in dilution water was conducted. The following preliminary study 2 was performed in fish acute toxicity test (Study number: 94228).

1) Preliminary study 1 for measurement of solubility

(1) Method

Since the test item was expected to volatile due to the chemical structure, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature (20±1°C) for 24 and 48 hours. And then the middle layer was sampled after settling for approx. 1 hour. Centrifugation and filtration with a membrane filter for removal of insoluble substance were not demonstrated, because these methods caused the decrease of test item concentration. The concentration of the test item in the collected sample was analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring hours	48-hour stirring
Approx. 100 (Sample-1)	0.0400	-
Approx. 100 (Sample-2)	0.0351	-
Approx. 100 (Sample-3)	-	0.0404
Approx. 100 (Sample-4)	-	0.0324

Solubility of test item in dilution water was around 0.03 to 0.05 mg/L.

2) Preliminary study 2 for measurement of solubility

(1) Method

Since the test item was forecasted to be hydrolyzed into 13F-EtOH, the solubility of the test item in dilution water and 13F-EtOH (the test item of study number 94232-94234) were measured at the same time. Firstly, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature (24±1°C) for 24 and 48 hours. And then the middle layer was sampled after settling for approx. 1 hour. The concentration of the test item and 13F-EtOH in the collected sample were analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

Test item

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring hours	48-hour stirring
Approx. 100 (Sample-1)	0.0218	-
Approx. 100 (Sample-2)	0.0303	-
Approx. 100 (Sample-3)	-	0.0351
Approx. 100 (Sample-4)	-	0.0221

13F-EtOH

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring hours	48-hour stirring
Approx. 100 (Sample-1)	0.0157	-
Approx. 100 (Sample-2)	0.0162	-
Approx. 100 (Sample-3)	-	0.0364
Approx. 100 (Sample-4)	-	0.0283

The solubility of the test item in dilution water was around 0.02 to 0.04 mg/L, and the measured concentrations stirred for 24 and 48 hours were about the same value. The measured concentration of 13F-EtOH (hydrolyzed product) was 0.01 to 0.02 mg/L at 24-hour stirring, and 0.02 to 0.04 mg/L at 48-hour stirring.

3) Summary of preliminary study for measurement of solubility

Since the test item was expected to volatile due to the chemical structure, the test solution was stirred gently in a devised glass container under closed system with no head space. By the results of preliminary study 1, the solubility of the test item in dilution water was around 0.03 to 0.05 mg/L, but by the result of preliminary study 2, it was no more than 0.02 to 0.04 mg/L in dilution water. It was considered that the solubility of the test item in dilution water was around 0.01 to 0.1 mg/L with consideration of the stirred condition, properties of test item and concentration level. On more by the results of preliminary study 1 and 2, there was no difference between the solubility of the test item prepared by stirring for 24 and 48 hours, but the measured concentration of 13F-EtOH (hydrolyzed product) at 48-hour stirring was twice as much as that at 24-hour stirring.

From the results mentioned above, in definitive study the devised glass container would be used for the preparation in definitive study. It was decided that the test solution was settled for 24 hours, because the solubility of the test item at 24 and 48-hour stirring were about the same value, and the production amount of hydrolyzed product was low.

2. Study for effect on test organism

1) Preliminary study

(1) Method

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for approx. 48 hours under approx. 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. The preliminary study to investigate the effect of the test item on the test organisms was performed under closed system, and static and semi-static regime (renewal after 24 hours). The measurement of the test item in the test solution was also carried out. The test sample was employed in terms of volume using the density [1.496 g/cm^3(25°C)] for the preparation of test solution.

(2) Result

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Others	Immobility (%)	Others
100 (Static)	0	-	0	-
100 (Semi-static)	0	-	0	-

Number of organism: Ten daphnids/test level (five daphnids/replicate),

Closed system

- shows that no other abnormal response was observed.

Nominal concentration (mg/L)	Measured concentration (mg/L) (percentage of measured concentration at start)		
	At the start	after 24 hours	At the end (after 48 hours)
100	0.0349	0.0215 (61.7)	0.0173 (49.6)

No effect on the test organisms was observed in both static and semi-static regime. The measured concentration of the test item at the start of the exposure was around the solubility in the dilution water, but it gradually decreased with time.

2) Summary of effect on test organisms (preliminary study)

The test sample and the dilution water were mixed and stirred for 48 hours to produce a limit concentration (100 mg/L) in "Testing Methods for New Chemical Substances" , and the prepared test solution which was taken out from the middle layer of the dispersed solution had no effect on the test organisms. Since the test item was suspected to volatile, the investigation was performed with closed system. However, the measured concentration of the test item decreased during the exposure, therefore, the definitive study was planed to be conducted with semi-static replacement regime.

3. Result of preliminary study (Summary)

In the preliminary study for measurement of solubility, the concentration of the test item in the dilution water was supposed to reach the saturated value by stirring for 24 hours. The hydrolyzed product (13F-EtOH) of the test item gradually increased with time.

In the preliminary study for effect on test organism, no adverse effect was observed in the saturated solution prepared with 48-hour stirring of general procedure. However, while both the concentrations prepared by 24-hour and 48-hour stirring were almost the same value as the solubility obtained in the preliminary study for measurement of solubility, the hydrolyze product was produced more in the saturated solution at 48- hour stirring than that at 24-hour. Therefore, I thought that it was proper to use the test solution containing less concentration of the hydrolyzed product in the case of the similar concentration in the saturated solution of the test item, and then the definitive study planed to conduct using the saturated test solution prepared by 24-hour stirring. It was expected that no effect of the test item on the organisms would be observed in the saturated test solution prepared by 24-hour stirring.

4. Operation of definitive study

1) Measurement of solubility of test item in dilution water

Based on the result of the preliminary study, the measurement of the solubility was carried out using the solution prepared by mixing the test sample and the dilution water to produce approx. 100 mg/L, and by stirring gently for 24 hours under closed system and 20±1°C. For removal of insoluble substance, the procedure of centrifugation and filtration was not employed. Instead of using their procedure, to minimize insoluble substance it was removed by taking out from the middle layer of the solution settled for approx. 1 hour after cease of stirring. The concentration of the test item and 13F-EtOH, which was the hydrolyzed product of the test item, was measured for the prepared test solution.

2) Definitive study

Since the preliminary study resulted in no effect of the test item on the test organisms at the concentration around the solubility in the dilution water, the definitive study was planed to be carried out under closed system using the nominal test concentration of an upper limit (100 mg/L) of exposure level in the test method, and using the middle layer of the test solution prepared by stirring for approx. 24 hours. The test solution was prepared as follows; after the test sample was added, in terms of volume using the density, into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to produce 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for approx. 24 hours under approx. 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for approx. 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. No correction of purity was employed for the preparation of the test solution. The measurement of the test item and 13F-EtOH in the test solution was carried out at the start of the exposure, before and after the renewal, and at the end of the exposure.