CERI

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Study number	94224

FINAL REPORT

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

Study number 94224

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94224, issued on July 26, 2007) and reflects a correct English translation of corrected parts in the amendments of the Final Report (issued on August 23, 2007) audited by Quality Assurance Unit of Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan.

Date October 1, 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-SFA with *Daphnia magna*

Study number 94224

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-SFA with *Daphnia magna*

Study number 94224

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 1, 2007	June 1, 2007
Study plan	June 5, 2007	June 5, 2007
Amendment of study plan	July 25, 2007	July 26, 2007
Measurement of solubility	June 6, 2007	June 8, 2007
	June 7, 2007	June 8, 2007
Start of the exposure and after the exposure	June 5, 2007	June 8, 2007
	June 6, 2007	June 8, 2007
	June 8, 2007	June 8, 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

Title	A 48-hour Acute Immobilization Study of 13F-SFA 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-SFA 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 4, 2007
Experimental starting date	June 6, 2007
Solubility study starting date	June 6, 2007
Bioassay starting date	June 6, 2007
Experimental completion date	June 8, 2007
Solubility study completion date	June 7, 2007
Bioassay completion date	June 8, 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". Treatment of the test material after the storage period will be discussed with sponsor. If it is not stable for the storage period, it will be stored as long while it is kept stable and it is disposed with approval of sponsor.

*1 It will be stored as the common sample for storage of these studies (Study Nos. 94223, 94224 and 94225).

(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 :
Section 4 (Eco-toxicity test area)

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-SFA with *Daphnia magna*

Test conditions

(1)	Test item	13F-SFA
(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)	The 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 prepare 100 mg/L (nominal concentration), and they were stirred under closed system for approx. 24 hours. After settlement for approx. 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 13F-EtOH (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) 0.181 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 0.193 and 0.127 mg/L

Before the renewal and at the end of the exposure 0.157 and 0.103 mg/L (81.4 and 81.3%)

(3)Concentration of 13F-EtOH in test solution

At the start of the exposure and after the renewal 0.0178 and 0.0126 mg/L

Before the renewal and at the end of the exposure 0.0459 and 0.0208 mg/L

(4)48-hour EC_{50} (Median Effective Concentration) > 0.141 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

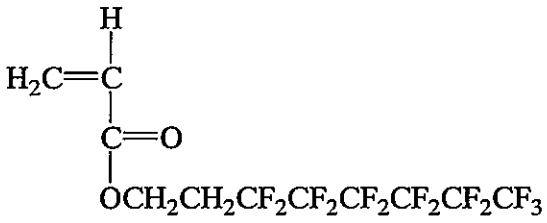
In this report, 13F-SFA has the following name etc.

1.1 Chemical name[*2]

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

1.2 Chemical structure etc.[*2]

Structural formula



Molecular formula	$C_{11}H_7F_{13}O_2$
Molecular weight	418.15
CAS Number	17527-29-6

*2 Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number*2

Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	6X002

2.2 Purity*2

Test item	99.7%	
Impurity	Unknown constituent component	0.3%

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	78°C (8 mmHg)	
Density	1.554 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 14-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 April 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 using 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 of 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) The number of organisms

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 60% of the saturation at the test temperature. 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 [1.554 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 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. 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, and not more than 10% of the control daphnids should show trapping at surface of water.

(2) Dissolved oxygen concentration should be more than 60% of the saturation at the test temperature.

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 8.3 to 8.4 mg/L, 7.7 to 7.8 and 19.8 to 20.2°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 60% of the saturation*4 at the test temperature).

*4 Saturated concentration at 19 to 21°C: 9.01 to 8.68 mg/L [JIS K 0102: 1998]

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.193 and 0.127 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.157 and 0.103 mg/L which were 81.4 and 81.3% of the concentration at the preparation. The measured concentrations of 13F-EtOH in the test solution were 0.0178 and 0.0126 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.0459 and 0.0208 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-SFA to *Daphnia magna* were >0.141 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 (0.127 mg/L) compared with the solubility (0.181 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 0.124 mg/L was in them. The measured concentrations at 24 hours after the preparation decreased but not so much as it was predicted from the result of the preliminary studies. Since no adverse effect was found 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 test solution, and the measured concentrations were 0.0126 to 0.0459 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*5 (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	
0.141	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	

*5: 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	-	-
0.141	-	-

- : 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	8.3	8.3	8.3	8.3
0.141	8.4	8.4	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	7.8	7.8	7.8	7.7
0.141	7.8	7.8	7.8	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	20.0	19.9	19.9	19.8
0.141	20.2	19.9	19.9	19.8

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	>0.141	- (-)	-
48-hour	>0.141	- (-)	-

- : 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 and13F-EtOH (hydrolyzed product)

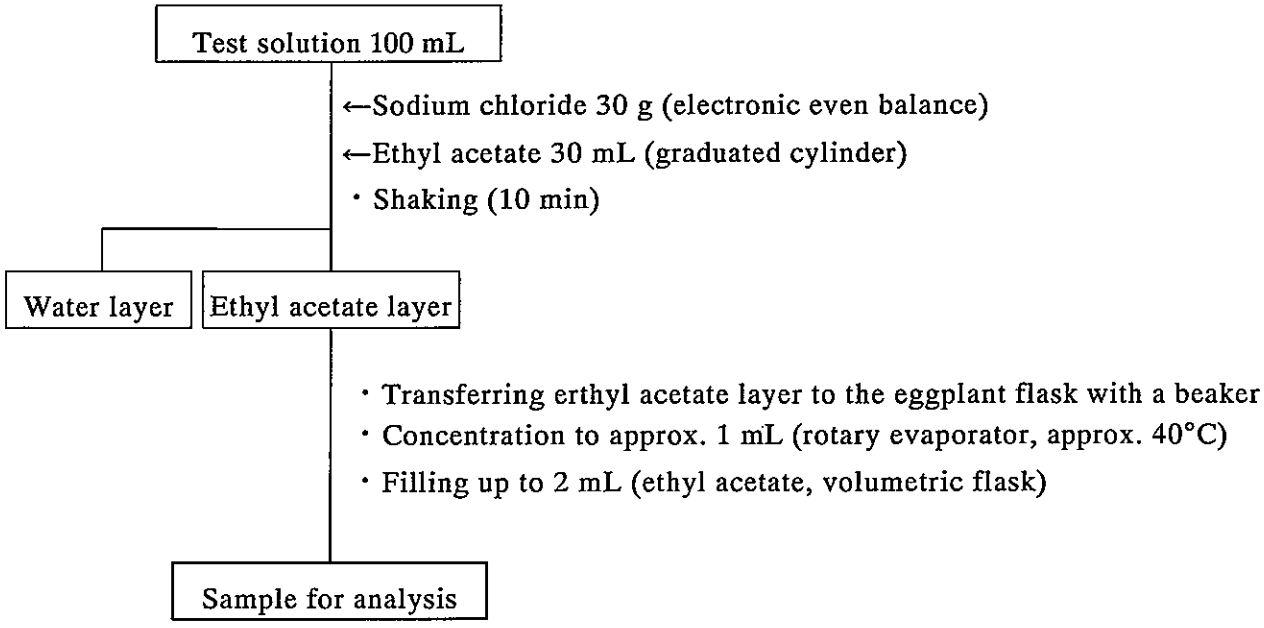
1. Analysis of test solution

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



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 (99.7%) of the test item and the purity (99.8%) of 13F-EtOH.

The test sample of 100.3 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 0.00606 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 0.00604 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

81.9%, 83.0% average 82.5%

Recovery rate of 13F-EtOH for pretreatment

82.6%, 83.0% average 82.8%

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 of test item (mg/L) (Percentage of measured concentration versus that at each preparation %)				
	At the start	24 hours		At the end	Geometric mean
		Before the	After the renewal		
Control	n.d.	n.d.	n.d.	n.d.	
100	0.193	0.157 (81.4)	0.127	0.103 (81.3)	0.141

n.d. : <0.00606 mg/L

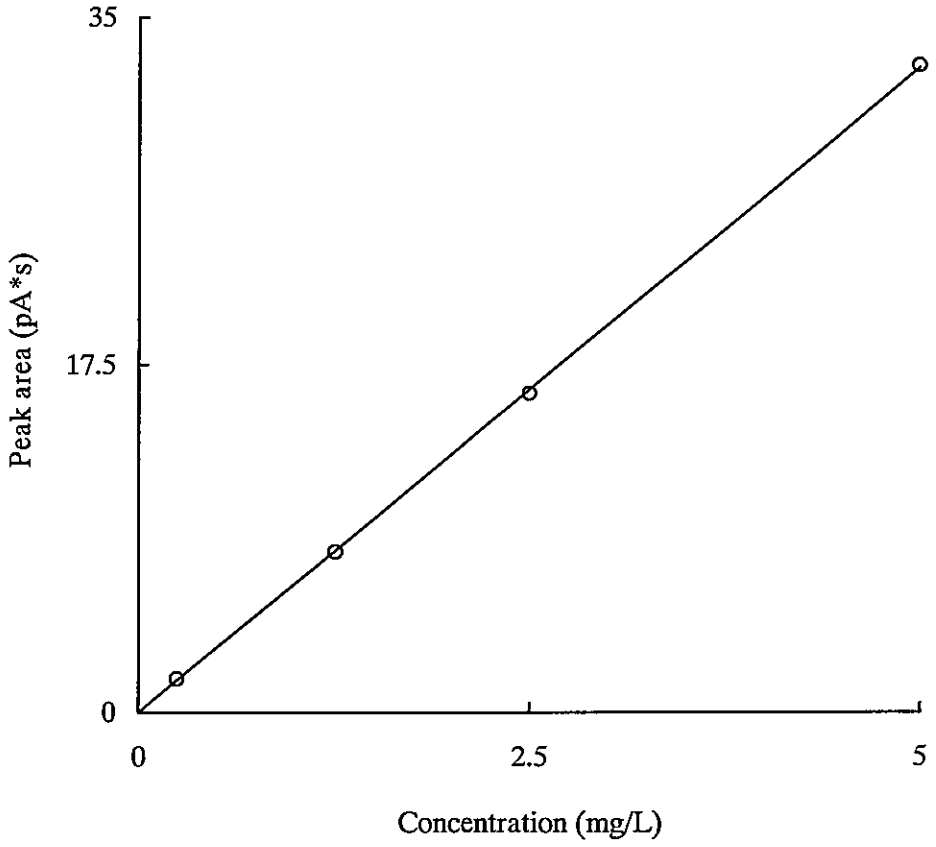
Appendix table 2-2 Measured concentrations of 13F-EtOH in test solutions

Nominal concentration (mg/L)	Measured concentration 13F-EtOH (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	0.0178	0.0459	0.0126	0.0208

n.d. : <0.00604 mg/L

Appendix 3

Calibration curve and chromatogram



y = 6.49x
r = 1.00

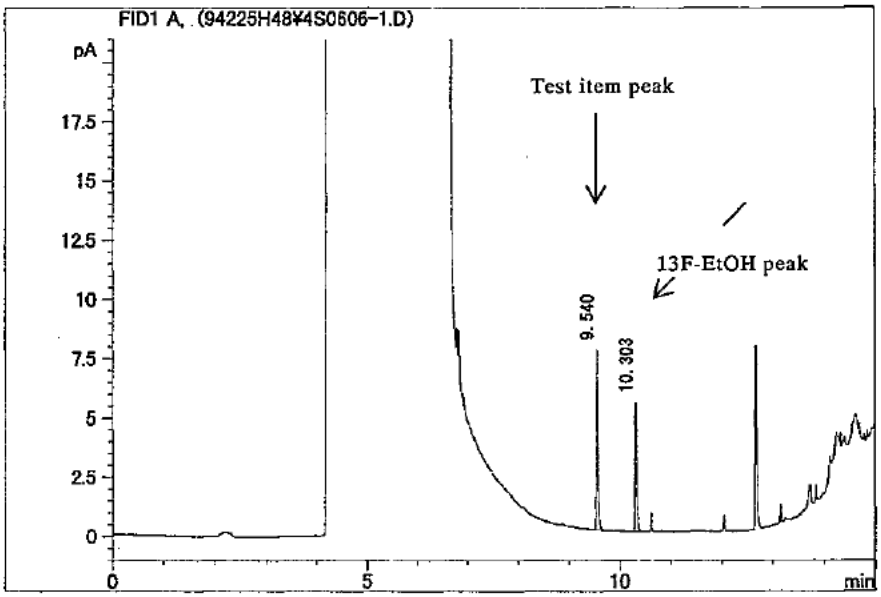
Concentration (mg/L)	Peak area (pA*s)
0.250	1.667
1.25	8.062
2.50	16.015
5.00	32.546

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

Standard solution 2.50 mg/L

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H48¥4S0606-1.D
サンプル名 : std 2.50mg/L



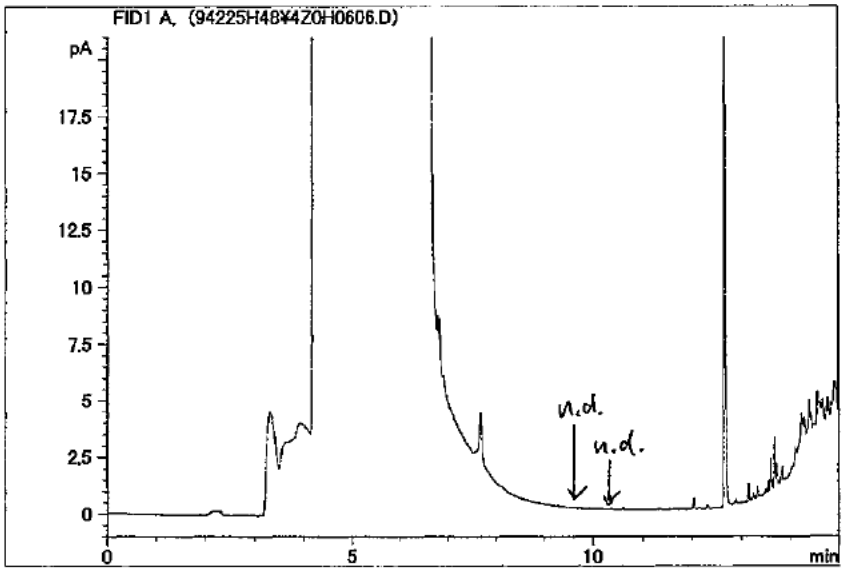
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	16.489	7.618	9.540
2	12.199	5.468	10.303

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

Control

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥94225H48¥4Z0H0606.D
サンプル名 : Cont. (*50) H0h



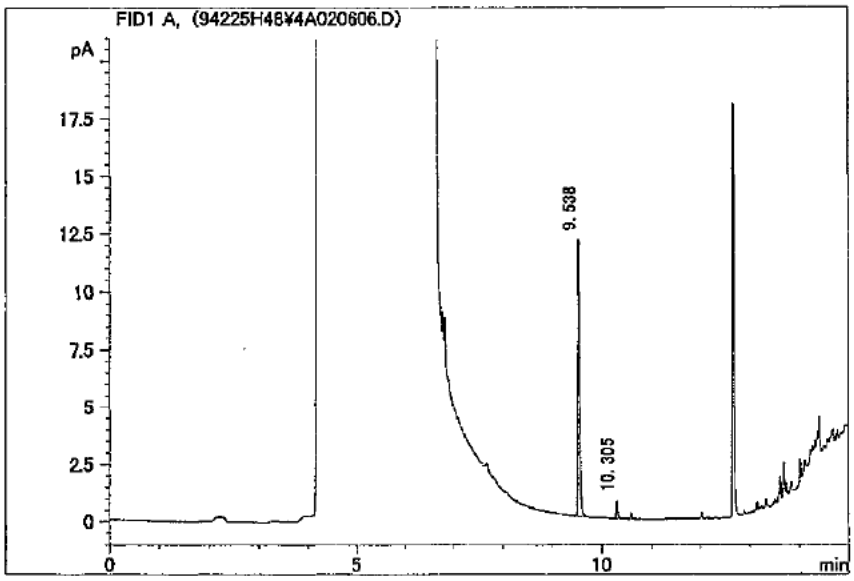
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

100 mg/L (Nominal concentration)

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H48¥4A020606.D
サンプル名 : 100(*50D2)H0h



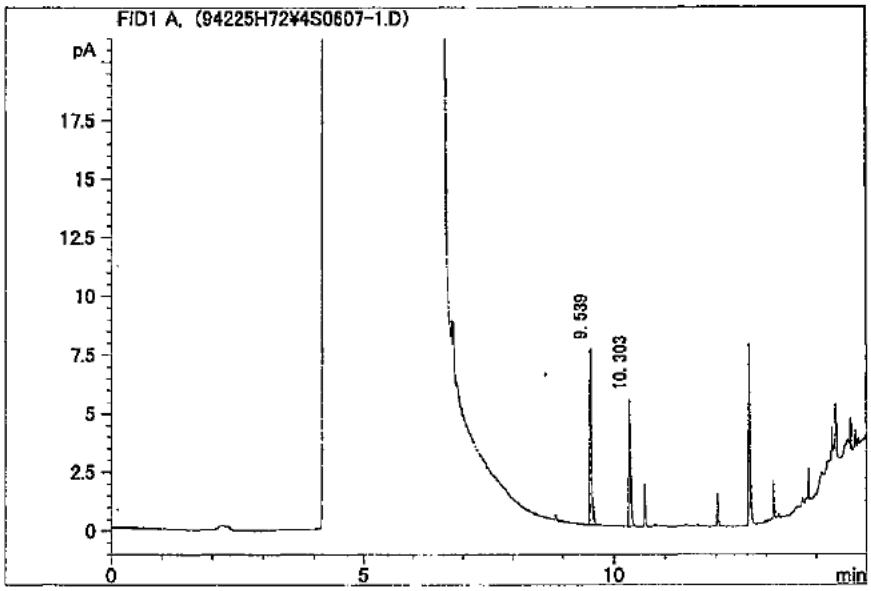
#	メインピーク面積	メインピーク高さ	実測 RT
1	26.286	12.011	9.538
2	1.817	0.792	10.305

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

Standard solution 2.50 mg/L

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥94225H72¥4S0607-1.D
サンプル名 : std 2.50mg/L



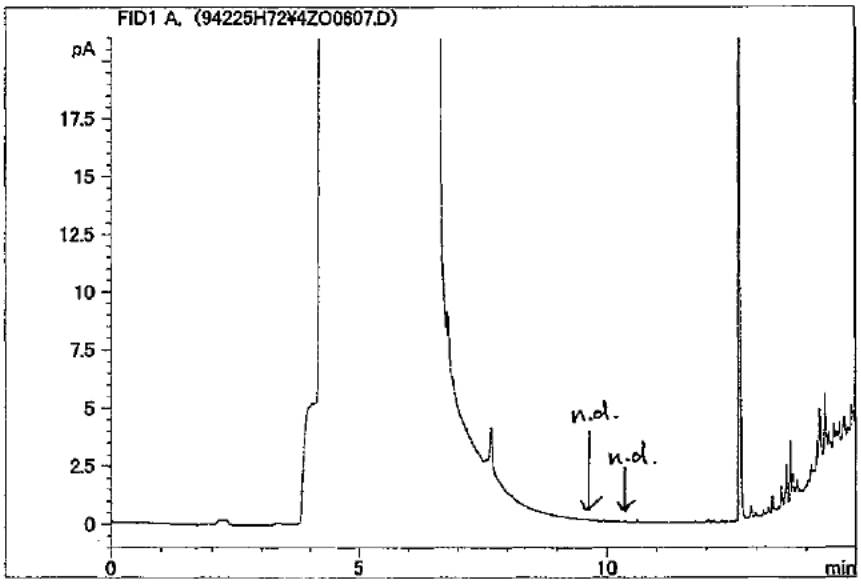
#	メインピーク面積	メインピーク高さ	実測 RT
1	16.204	7.510	9.539
2	12.062	5.420	10.303

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

Control

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥94225H72¥4ZO0607.D
サンプル名 : Cont. (*50) H24old



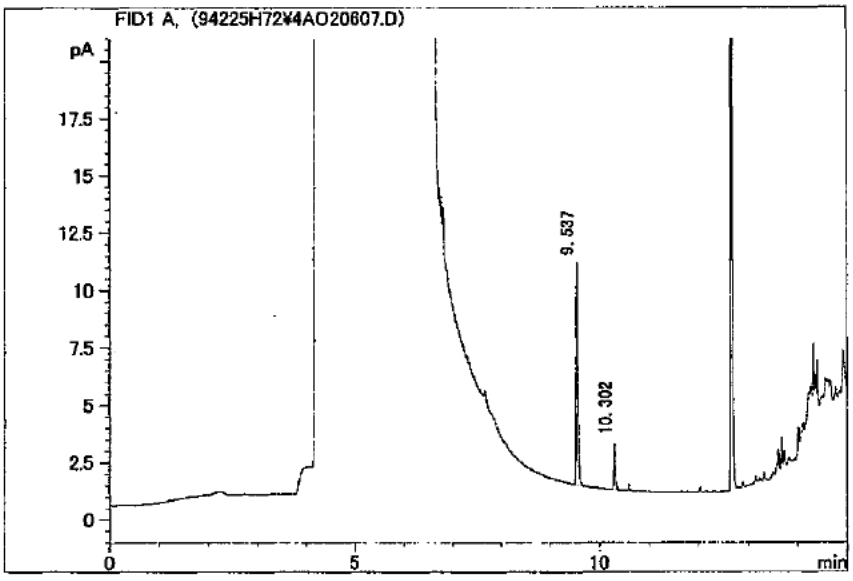
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

100 mg/L (Nominal concentration)

Study No. 94224

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94223.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥94225H72¥4AO20607.D
サンプル名 : 100(*50D2)H24old



#	メインピーク面積	メインピーク高さ	実測 RT
1	21.020	9.614	9.537
2	4.471	2.013	10.302

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

Appendix 4

Solubility 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)
Mixing apparatus	:Magnetic stirrer
Vessel	:Devised glass container (Interior volume : approx. 600 mL)

4.2 Test conditions

(1) Test temperature :20±1°C

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

(3) Dilution water :Dechlorinated tap water

(4) Repetition :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 (38.6 μL) was caluculated from the density of the test item (1.554 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 0.181 mg/L. In addition, the measured concentration of 13F-EtOH was 0.0154 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	0.124	0.181
Sample-2	0.161	
Sample-3	0.258	

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	0.0138	0.0154
Sample-2	0.0148	
Sample-3	0.0178	

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 algae growth inhibition test (Study number: 94223).

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 about 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	48-hour stirring
Approx. 100 (Sample-1)	0.286	–
Approx. 100 (Sample-2)	0.160	–
Approx. 100 (Sample-3)	–	0.161
Approx. 100 (Sample-4)	–	0.188

Solubility of test item in dilution water was around 0.1 to 0.3 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) as condition of fish acute toxicity test for 24 and 48 hours. And then the middle layer was sampled after settling for about 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	48-hour stirring
Approx. 100 (Sample-1)	0.392	-
Approx. 100 (Sample-2)	0.609	-
Approx. 100 (Sample-3)	-	0.0923
Approx. 100 (Sample-4)	-	0.0856

13F-EtOH

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	24-hour stirring	48-hour stirring
Approx. 100 (Sample-1)	0.0843	-
Approx. 100 (Sample-2)	0.0721	-
Approx. 100 (Sample-3)	-	0.552
Approx. 100 (Sample-4)	-	0.291

Higher concentration of the test item was obtained as solubility at 24-hour stirring than that in the preliminary study 1. However, it was though to be due to addition of suspended test item because the vessel for stirring was a devised glass container but Erlenmeyer flask. 13F-EtOH, a hydrolyzed product of the test item, was produced at concentration of 0.07 to 0.1 mg/L at 24-hour stirring and 0.2 to 0.6 mg/L at 48-hour stirring.

3) Summary of preliminary study for measurement of solubility

Since the test item was suspected to volatile, the preparation of saturated solution for the measurement of the solubility was employed using a devised glass container and gentle agitation. The solubility of the test item in the dilution water was around 0.1 to 0.3 mg/L based on the result of the preliminary study 1. The measured concentrations at 24-hour and 48-hour stirring were within 2 times, and then they were supposed to be almost same concentration based on judging from the order of the concentration and the characteristics of the test item. In the preliminary study 2 for measurement of solubility, the produced concentration of 13F-EtOH was approximately 0.08 mg/L at 24-hour stirring, while it

remarkably increased to 0.2 to 0.6 mg/L at 48-hour stirring.

From the results mentioned above, it was concluded that the devised glass container would be used for the preparation, and the procedure of 24-hour stirring, where the solubility of the test item reached to more than the same solubility as that in 48-hour stirring and also the hydrolyzed product was fewer, would be employed in the definitive study.

2. Study for effect on test organism

1) Preliminary study 1

(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 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. The preliminary study to investigate the effect of the test item on the test organisms was performed under static and semi-static regime (renewal after 24 hours). The measurement of the test item in the test solution was also carried out.

(2)Result

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Others	Immobility (%)	Others
100 (Static)	60	RA	90	-
100 (Semi-static)	50	-	100	-

The number of organisms: Ten daphnids/test level (five daphnids/replicate) with closed system

- shows that no other abnormal response was observed.

Abbreviation

RA: Reduced activity

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	2.10	0.440 (21.0)	0.172 (8.18)

90 and 100% immobility were observed in static and semi-static regime, respectively. In addition to the abnormal response, adhesion of the test item to the body surface of daphnids was observed. The measured concentration of the test item at the start of the exposure was approximately 10 times of the solubility (0.1 to 0.3 mg/L), which was obtained in the preliminary study 1, in the dilution water. This was thought to be due to containing the dispersed particle of the test item, which couldn't be removed by the settlement for approx. 1 hour, in the test solution taken out from the middle layer, and then the suspended particle might affect the test organisms physically.

2) Preliminary study 2

(1) Method

It was thought that the reason why the insoluble substance couldn't be removed well was due to stirring vigorously where more revolution per minute was employed than that in preliminary study for measurement of solubility. In this preliminary study, the investigation was carried out to confirm whether or not the saturated test solution could be prepared with the method of a gentle stirring (approx. 250 rpm) such as used in measurement of solubility. Additionally, a bit higher energy (approx. 500 rpm) than it was investigated.

The preliminary study to investigate the effect of the test item on the test organisms was performed using a gentle stirring (approx. 250 and 500 rpm) under static and semi-static regime (renewal after 24 hours). The test sample was employed in terms of volume using the density [1.554 g/cm^3(25°C)] for the preparation of test solution of 100 mg/L as nominal concentration. The measurement of the test item in the test solution was also carried out.

(2) Result

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Others	Immobility (%)	Others
100 (500rpm, static)	0	-	0	-
100 (500rpm, semi-static)	0	-	0	-
100 (250rpm, static)	0	-	0	-
100 (250rpm, semi-static)	0	-	0	-

The number of organisms: Ten daphnids/test level (five daphnids/replicate) with 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 (500rpm)	0.135	0.0704 (52.2)	0.0197 (14.6)
100 (250rpm)	0.103	0.0450 (43.6)	0.0118 (11.4)

No effect on the test organisms was observed in both studies under static and semi-static regime. The measured concentrations of the test item in the test solutions prepared with the different revolution per minute didn't show such value as greatly exceed the solubility. Since they were around the solubility, it was thought that no suspended test item contained in the prepared test solution. The measured concentration of the test item in the test solution prepared by 500 rpm was much closer than that by 250 rpm. The measured concentration of the test item in the test solution decreased gradually during the exposure.

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

The test sample and the dilution water were mixed and stirred for approx. 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, and a control. The test solution was prepared as follows; After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter 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 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 concentration. 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.