CERI

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Study number	94228

FINAL REPORT

A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka

July 25, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka

Study number 94228

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94228, issued on July 25, 2007)

Date September 10, 2009

Study Director

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka

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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 25, 2007

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka

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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 15, 2007	June 15, 2007
Study plan	June 18, 2007	June 18, 2007
Measurement of solubility	June 18, 2007	June 20, 2007
	June 19, 2007	June 20, 2007
Start of the exposure and after the exposure	June 18, 2007	June 22, 2007
Final report	June 19, 2007	June 22, 2007

Date July 25, 2007

Head of Quality Assurance Unit Signed in original

CONTENTS

Title	A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka
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 purpose of this study is to determine the acute toxicity of 13F-SFMA on fish.
Test method	This study was performed according to the following test methods and guidance document. (1) Fish, Acute Toxicity 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, 203 Fish, Acute Toxicity Test (Guideline 203, 1992) (3) OECD Guidance Document No.23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substance 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 15, 2007
Experimental starting date	June 18, 2007
Solubility study starting date	June 18, 2007
Bioassay starting date	June 18, 2007
Experimental completion date	June 22, 2007
Solubility study completion date	June 19, 2007
Bioassay completion date	June 22, 2007
Study completion date	July 25, 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 25, 2007
	Signature	Signed in original

SUMMARY

Title

A 96-hour Acute Toxicity Study of 13F-SFMA with Medaka

Test conditions

(1) Test item	13F-SFMA
(2) Test organism	Medaka (*Oryzias latipes*)
(3) Exposure duration	96 hours
(4) Test concentration	The solution which collected from the middle layer in the suspension of the test item (nominal concentration: 100 mg/L) and control
(5) Replicate	Two replicates/test level
(6) Number of organism	Ten fish / test level (five fish / test vessel)
(7) Dilution water	Dechlorinated tap water
(8) Type of test	Closed Semi-static (renewal at every 24 hours)
(9) Preparation of test solution	The test sample and dilution water were mixed to produced about 100 mg/L (nominal concentration) and they were stirred under closed system for about 24 hours. After settlement for 1 hour, test solution was prepared by taking out from the middle layer.
(10) Volume of test solution	About 6 L / test level (About 3 L / test vessel)
(11) Temperature of test solutions	24±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 of test item in dilution water (24±1°C)	0.0399 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	0.137 to 0.593 mg/L
Before the renewal and at the end of the exposure	0.0302 to 0.126 mg/L (14.3 to 27.8%)
(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	0.0203 to 0.0876 mg/L
(4) 96-hour LC_{50} (Median Lethal Concentration)	> 0.133 mg/L

[The values of (4) is based on 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

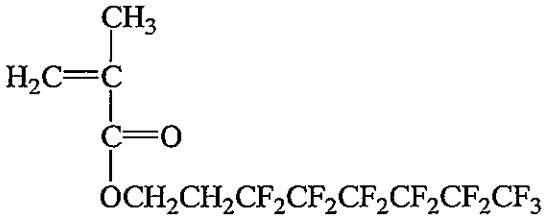
In this report, 13F-SFMA has the following name etc.

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

Medaka (*Oryzias latipes*)

(2) Reason for selection of species

This species is recommended in the test guidelines.

(3) Size

Total length 2.3±1.2 cm

Size of test organism was applied the regulated size set to test method (1).

(4) Supplier

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan

3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan

(5) Acclimation

Medaka hatched out on December 11, 2006 were acclimated for 28 days by flow-through condition under the same water quality (dechlorinated tap water), temperature (24±1°C), light and dark period (16-hour light / 8-hour dark) as test condition (on May 21 to June 18, 2007). The mortality was 0% during the 7 days before the start of the exposure. The test organism at the start of the exposure was 6-month-old fish. The test organisms were not treated with a medicament for external disinfection. The test organisms were fed the feed mixture for carp (2C), and not fed for 24 hours before the start of the exposure. Dissolved oxygen concentration in breeding water during acclimation was kept not less than 80% of air saturation value. A 96-hour acute toxicity test of $CuSO_4$ $5H_2O$ (Reagent chemical, Wako Pure Chemical Industries, Ltd.) to confirm reproducibility of the test system was carried out on May 21 to May 25, 2007 and the 96-hour LC_{50} was 0.445 mg/L. This value was within the stipulated range (mean ± 2S.D. : 0.124 to 0.978 mg/L) [mean ± S.D. : 0.551 ± 0.214 mg/L (n=38)] to background data in this laboratory. All of the values shown above for the reference substance were converted into $CuSO_4$ value.

(6) Allocation to the test groups

Medaka were allocated at random to each test group.

3.2 Dilution water

Dechlorinated tap water, aerated sufficiently and controlled temperature, 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 : 3 L Glass tank (diameter: 16 cm, depth: 17 cm)

The test vessels were covered with lid in order to prevent dust, and volatilization of the test solution and closed without headspace.

(2) Test equipment

Water bath : Plastic tank

Warming / cooling unit (Type HCA 250, 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 solutions were renewed at every 24 hours, as closed semi-static regime.

(b) Exposure duration

96 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 effect on the test organisms. 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 manner as the test solution, was used as the control.

(e) Replicates

Two replicates / test level

(f) Number of organism

Ten fish / test level (five fish / test vessel)

(g) Volume of test solution

About 6 L / test level (about 3 L / test vessel)

(2) Conditions of test environment

(a) Water temperature

24±1°C

(b) Dissolved oxygen concentration

The test was performed in the condition where dissolved oxygen concentration was at least 60% or more of the saturated concentration at the water temperature. Aeration was not used 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 about 24 hours 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

Mortality and visible abnormality were observed at 3, 24, 48, 72 and 96 hours after the start of the exposure. A fish was considered as dead if the observable motion (motion of mouth and opercula etc.) were not observed and touching of the caudal peduncle with glass rod produced no reaction.

(2) Total length and body weight of test organism

The test organisms in the control group were used for measuring total length and body weight after the end of the exposure.

(3) Appearance of test solution

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

(4) 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 the end of the exposure. At the preparation, another solution sampled from the container for preparation was used for the measurement. At 24 hours after, 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 Incorporated., YSI Model 58). The pH measurements were carried out with a portable pH meter (DKK-TOA, Model HM-21P). The temperature measurements were carried out with a calibrated red alcohol thermometer of glass stick type.

(5) 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. 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 and after the renewal (at the preparation) another solution sampled from the container for preparation was used for analysis. Before the renewal and the end of the exposure, 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.

(6) 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 LC_{50} *3

The LC_{50} values were estimated as "> the test concentration" since no less than 50% of mortality was observed in the exposure level.

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

*3 LC_{50} (Median Lethal Concentration): The test item concentration at which 50% of the test organisms causes mortality during the exposure.

3.8 Validity of the test

(1) The mortality in the control should not exceed 10%.

(2) Dissolved oxygen concentration must be at least 60% of the air saturation value at the water temperature in the test during 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. Rcuesults and disssion

4.1 Mortality

The mortality during the exposure was not observed in the exposure level. Cumulative mortality of each exposure period was shown in Table 1. The mortality in the control was not observed, which meets the criterion for the validity of the test (i.e. not more than 10%).

4.2 Observed abnormal response

No abnormal responses were observed in the control.

The results of observation were based on the comparison with the control organisms. No abnormal responses were observed in the exposure level during exposure. The abnormal responses observed during the exposure are shown in Table 2.

4.3 Size of test organism [Mean ± Standard deviation (n=10)]

Total length	2.7±0.13 cm
Body weight	0.17±0.015 g

4.4 Observation and measurement of test solution

(1) Appearance of test solution

The test solutions were clear and colorless at the start of the exposure and before the renewal.

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature during the exposure ranged from 6.2 to 8.3 mg/L, 7.2 to 7.8 and 24.0 to 24.7°C, respectively. Conditions of the test solutions are shown in Tables 3-1, 3-2 and 3-3. The measured values of dissolved oxygen concentration met the criterion for the study validity (at least 60% or more of saturate concentration*4 at the water temperature).

*4 Saturated dissolved oxygen concentration (23 to 25°C): 8.39 to 8.11 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 0.137 to 0.593 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.0302 to 0.126 mg/L which were 14.3 to 27.8% 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, 0.0203 to 0.0876 mg/L at before the renewal and at the end of the exposure. The results of the measured concentrations of the test item and 13F-EtOH are shown in Appendix 2.

4.5 LC_{50}

Both of the 48 and 96-hour LC_{50}s of the test item to Medaka were >0.133/L.
The LC_{50}s at each time are shown in Table 4.

4.6 Discussion

This study was conducted as a limit test in order to confirm the effect of the test organisms at around the solubility of the test item in dilution water. Although all of the measured concentrations of the test item at the preparation during the exposure were over the solubility to dilution water, they were decreased at 24 hours after the preparation. It was presumed that the cause of the decrease was by volatilization of the test item, by advance the hydrolysis of the test item with time. Semi-static regime (renewal at every 24 hours) was chosen to maintain the concentration of the test item. Therefore, it was considered that this definitive study was appropriate as the test at around the solubility. In addition, the environmental conditions were also within the suitable range. Therefore, we evaluated that this study complied with the applied test guidelines. 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. On the other hand, the measured concentrations of 13F-EtOH which was hydrolyzed product of the test item were ranged from 0.0203 to 0.0876 mg/L in the test solution at before the renewal and the end of the exposure. 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 Cumulative mortality

Measured concentration*5 (mg/L)	Cumulative mortality (%)				
	3 hours	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0	0
0.133	0	0	0	0	0

*5 Geometric mean of the measured concentrations

(The followings are expressed as measured concentration)

Table 2 Observed abnormal response

Measured concentration (mg/L)	Result of observation (Left column: Number of affected fish/Total survival number, Right column: Symptom detail)									
	3 hours		24 hours		48 hours		72 hours		96 hours	
Control	0/10	N	0/10	N	0/10	N	0/10	N	0/10	N
0.133	0/10	N	0/10	N	0/10	N	0/10	N	0/10	N

N : Normal (No abnormal response)

Table 3-1 Dissolved oxygen concentration of test solutions

Measured concentration (mg/L)	0 hour	24 hours		48 hours		72 hours		96 hours
	At the start	Before the renewal	After the renewal	Before the renewal	After the renewal	Before the renewal	After the renewal	At the end
Control	8.0	6.4	8.1	6.8	8.1	6.9	8.0	6.8
0.133	8.3	6.2	8.0	6.2	8.3	6.6	8.1	6.4

Unit : mg/L

Table 3-2 pH of test solutions

Measured concentration (mg/L)	0 hour	24 hours		48 hours		72 hours		96 hours
	At the start	Before the renewal	After the renewal	Before the renewal	After the renewal	Before the renewal	After the renewal	At the end
Control	7.7	7.3	7.7	7.3	7.7	7.3	7.6	7.2
0.133	7.8	7.3	7.7	7.3	7.7	7.4	7.7	7.2

Table 3-3 Temperature of test solutions

Measured concentration (mg/L)	0 hour	24 hours		48 hours		72 hours		96 hours
	At the start	Before the renewal	After the renewal	Before the renewal	After the renewal	Before the renewal	After the renewal	At the end
Control	24.1	24.2	24.4	24.0	24.2	24.1	24.2	24.0
0.133	24.2	24.2	24.7	24.1	24.2	24.1	24.2	24.0

Unit: °C

Table 4 LC_{50} to Medaka

Exposure duration	LC_{50} (mg/L)	95% confidence interval (mg/L) (Slope of the dose-response curve)	Statistical procedure used for determination of LC_{50}
24-hour	>0.133	- (-)	-
48-hour	>0.133	- (-)	-
72-hour	>0.133	- (-)	-
96-hour	>0.133	- (-)	-

- : Not obtained

Appendix 1

Chemical characteristics of dilution water

Chemical characteristics 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)

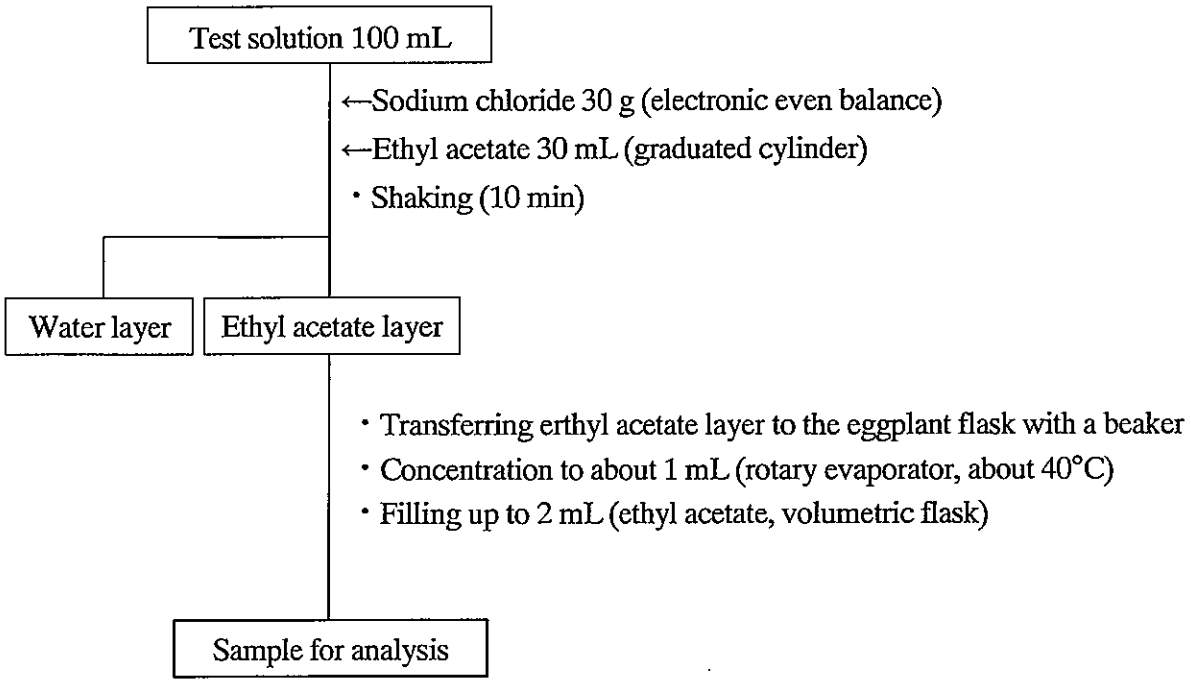
1. Analysis of test solution

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 (99.8%) 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		48 hours		72 hours		At the end	Geometric mean
		Before the renewal	After the renewal	Before the renewal	After the renewal	Before the renewal	After the renewal		
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
100	0.137	0.0302 (22.1)	0.593	0.126 (21.3)	0.561	0.0803 (14.3)	0.157	0.0437 (27.8)	0.133

n.d. : <0.00592 mg/L

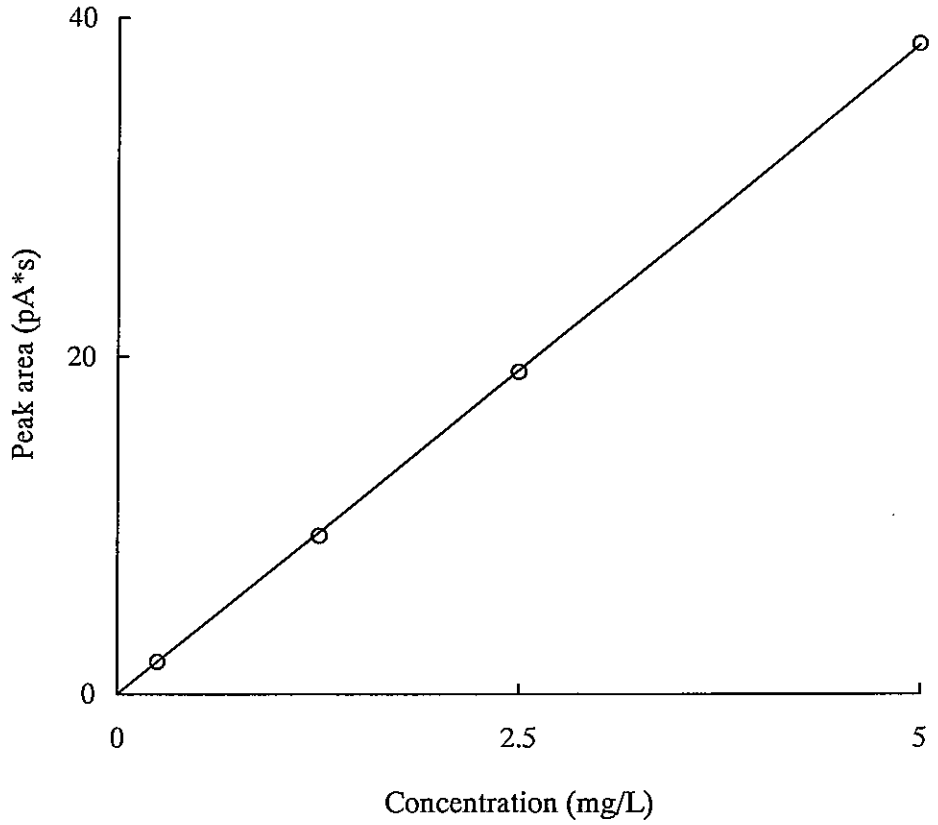
Appendix table 2-2 Measured concentrations of 13F-EtOH in test solutions

Nominal concentration (mg/L)	Measured concentration (mg/L)							
	At the start	24 hours		48 hours		72 hours		At the end
		Before the renewal	After the renewal	Before the renewal	After the renewal	Before the renewal	After the renewal	
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
100	n.d.	0.0203	n.d.	0.0876	n.d.	0.0435	n.d.	0.0318

n.d. : <0.00592 mg/L

Appendix 3

Calibration curve and chromatogram



y = 7.67x
r = 1.00

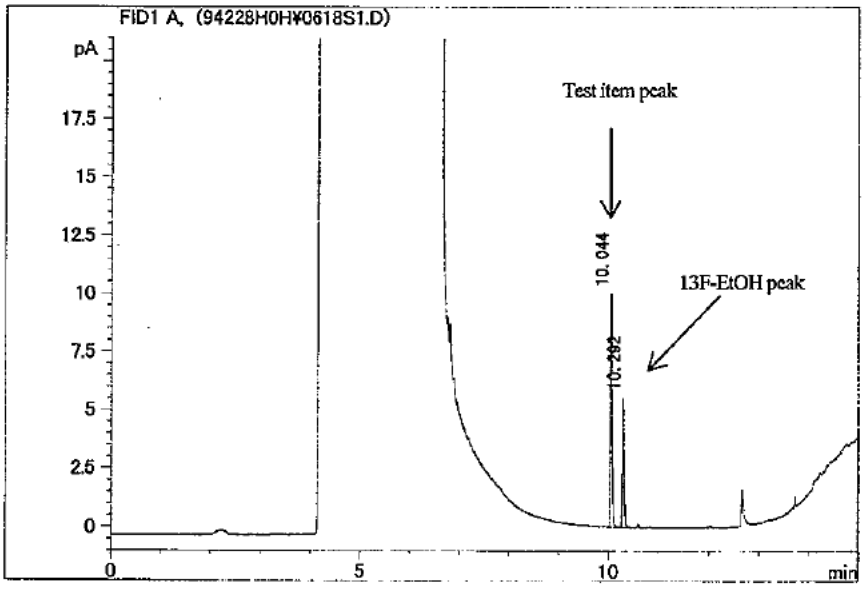
Concentration (mg/L)	Peak area (pA*s)
0.250	1.899
1.25	9.380
2.50	19.076
5.00	38.431

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

Standard solution 2.50 mg/L

Study No. 94228

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¥94228H0H¥0618S1.D
サンプル名 : std 2.50mg/L



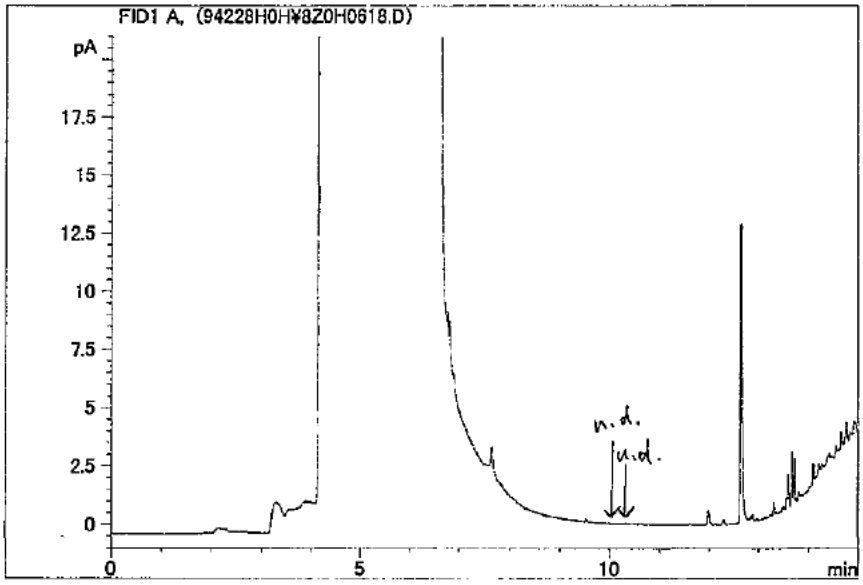
#	メインピーク面積	メインピーク高さ	実測 RT
1	19.175	9.936	10.044
2	12.308	5.511	10.292

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

Control

Study No. 94228

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¥94228H0H¥8Z0H0618.D
サンプル名 : Cont. (*50)H0h



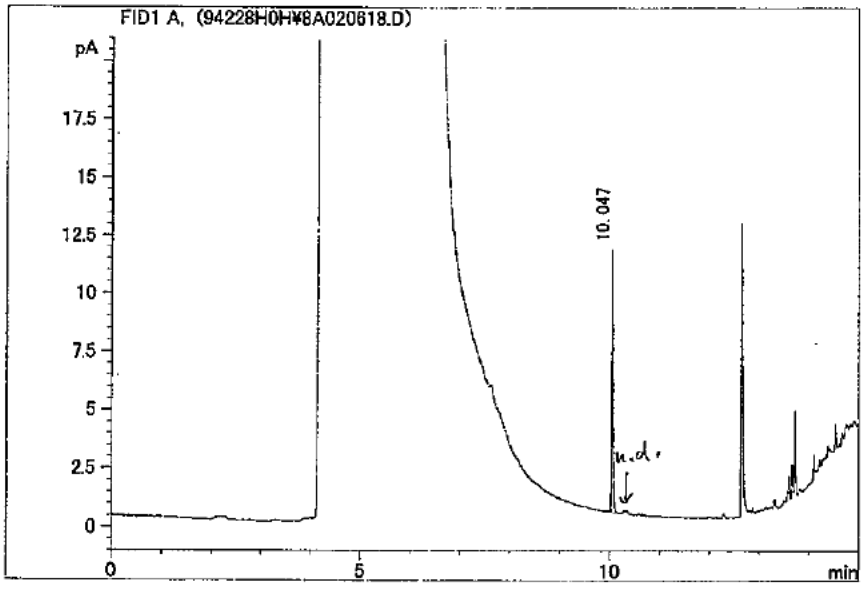
#	メインピーク面積	メインピーク高さ	実測 RT

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

100 mg/L (Nominal concentration)

Study No. 94228

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¥94228H0H¥8A020618.D
サンプル名 : 100(*50D2)H0h



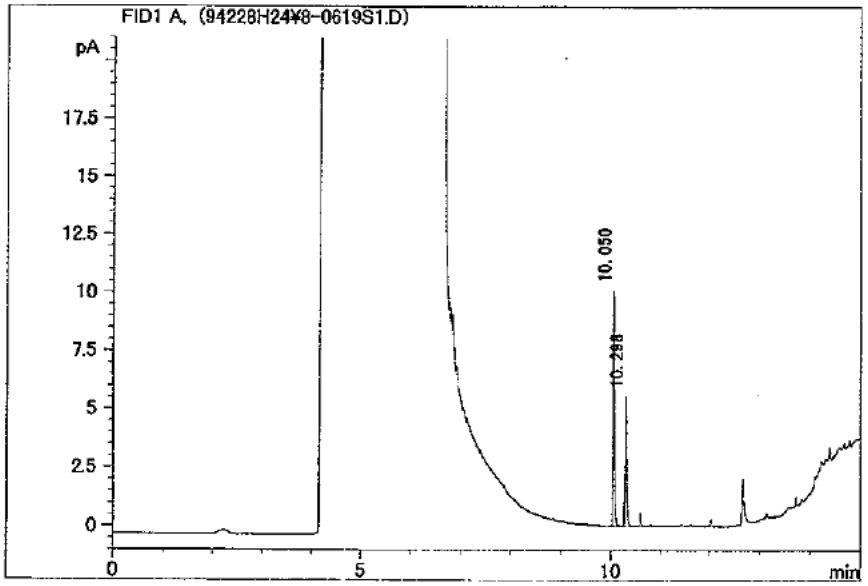
#	メインピーク面積	メインピーク高さ	実測 RT
1	22.120	11.240	10.047

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

Standard solution 2.50 mg/L

Study No. 94228

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¥94228H24¥8-0619S1.D
サンプル名　: std 2.50mg/L



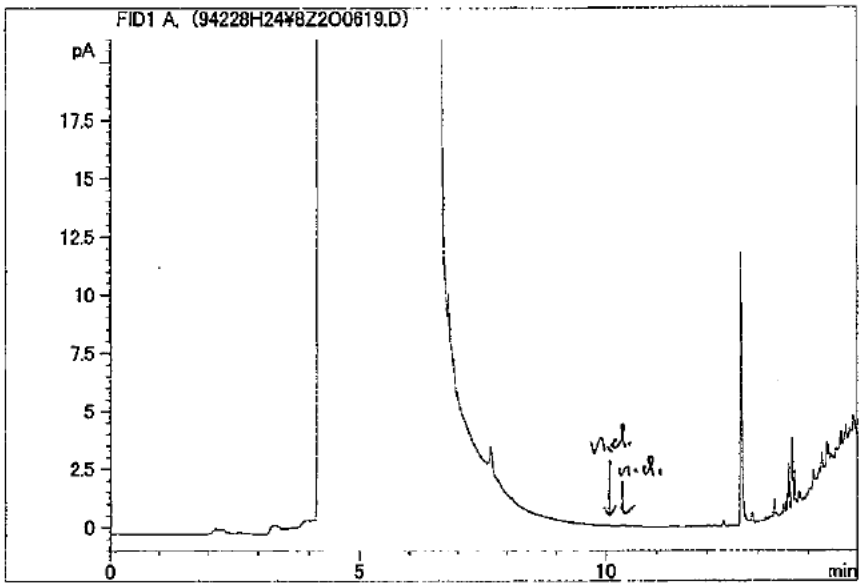
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.148	10.049	10.050
2	12.373	5.542	10.298

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

Control

Study No. 94228

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¥94228H24¥8Z200619.D
サンプル名 : Cont. (*50)H24old



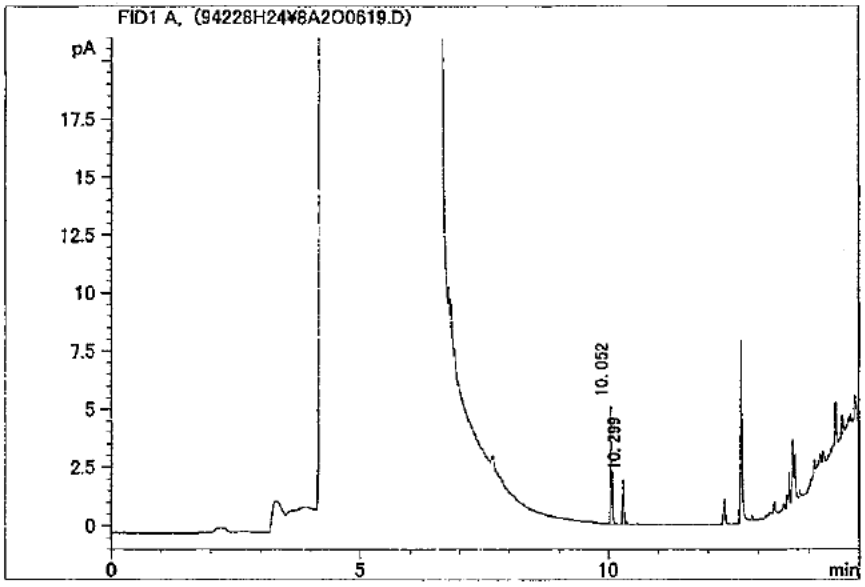
#	メインピーク面積	メインピーク高さ	実測 RT

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

100 mg/L (Nominal concentration)

Study No. 94228

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¥94228H24¥8A200619.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	9.765	5.066	10.052
2	4.236	1.902	10.299

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 : About 600 mL)

4.2 Test conditions

(1) Test temperature : 24±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 about 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 about 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 0.0399 mg/L. In addition, the measured concentration of 13F-EtOH was under 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	0.0560	0.0399
Sample-2	0.0317	
Sample-3	0.0321	

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.

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 (24±1°C) for 24 and 48 hours. And then the middle layer was sampled after settling for about 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)	
	Stirring for 24 hours	Stirring for 48 hours
Approx. 100 (Sample-1)	0.0773	-
Approx. 100 (Sample-2)	0.0399	-
Approx. 100 (Sample-3)	-	0.0324
Approx. 100 (Sample-4)	-	0.0167

Solubility of test item in dilution water was around 0.01 to 0.08 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 about 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

Measured concentration of the test item

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

Measured concentration of 13F-EtOH

Nominal concentration (mg/L)	Measured concentration (mg/L)	
	Stirring for 24 hours	Stirring for 48 hours
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 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 0.01 to 0.08 mg/L, but it 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 result 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 solubilities 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. 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 produce 100 mg/L as nominal concentration, the flask was immediately sealed with a plug to produce without head space. The solution was gently stirred by magnetic stirrer for about 48 hours to prepare the dispersed solution with suspended test item. This preliminary study was conducted by closed system without head space, since the test item was suspected to volatile. The saturation concentration of the test item in the test solution was also carried out.

(2) Result

Nominal concentration (mg/L)	Left column : Cumulative mortality (%) Right column : Existence of abnormal response (abnormalities : *, no abnormalities : -)									
	3 hours		24 hours		48 hours		72 hours		96 hours	
Control	0	-	0	-	0	-	0	-	0	-
100	0	-	0	-	0	-	0	-	0	-

Type of test : Semi-static (renewal at every 24 hours)

Number of organisms/volume of test solution: Five fish/about 3.4 L

Aeration: Not conducted

<Measured concentration of test item in test solution>

Nominal concentration (mg/L)	Measured concentration (mg/L) (percentage of measured concentration at start)	
	At the start	After 24 hours
Control	n.d.	n.d.
100	0.0550	0.0370 (67.1)

n.d. : <0.00607 mg/L

The saturation concentration of the test item in the test solution was decreased at 24 hours after the preparation.

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

No effect was observed on test organisms at the saturated solution of the test item which was prepared by mixing the test sample and dilution water and stirring. It was predicted that NOEC was over upper limit concentration

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) Preliminary study

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 approximately 100 mg/L, and by stirring gently for 24 hours under closed system and 24±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 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

The definitive study was conducted as a limit test with a control and the dispersed test solution prepared by mixing the test item and dilution water to produce upper limit concentration (100 mg/L) of test method, and stirring for about 24 hours. The definitive study was conducted by closed semi-static regime (renewal at every 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 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 about 24 hours 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 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.