Receipt number	662-06-E-4234
Study number	94234

FINAL REPORT

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

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 96-hour Acute Toxicity Study of 13F-EtOH with Medaka

Study number 94234

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94234, issued on July 26, 2007)

Date

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-EtOH 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 26, 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-EtOH with Medaka

Study number 94234

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 29, 2007	June 29, 2007
Study plan	June 29, 2007	June 29, 2007
Amendment of study plan	July 2, 2007	July 2, 2007
Measurement of solubility	July 4, 2007	July 9, 2007
	July 6, 2007	July 9, 2007
Start of the exposure and after the exposure	July 2, 2007	July 9, 2007
	July 6, 2007	July 9, 2007
Raw data and final report draft	July 25, 2007	July 25, 2007
Final report	July 26, 2007	July 26, 2007

Date July 26, 2007

Head of Quality Assurance Unit Signed in original

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Title	A 96-hour Acute Toxicity Study of 13F-EtOH 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-EtOH 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 29, 2007
Experimental starting date	July 2, 2007
Solubility study starting date	July 4, 2007
Bioassay starting date	July 2, 2007
Experimental completion date	July 6, 2007
Solubility study completion date	July 6, 2007
Bioassay completion date	July 6, 2007
Study completion date	July 26, 2007

Storage of test item, raw data, etc.

(1) Test item

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

*1 It will be stored as the common sample for storage of these studies (Study Nos. 94232, 94233 and 94234).

(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 96-hour Acute Toxicity Study of 13F-EtOH with Medaka

Test conditions

(1) Test item	13F-EtOH
(2) Test organism	Medaka (*Oryzias latipes*)
(3) Exposure duration	96 hours
(4) Test concentration	Five test concentrations of 60.0, 40.0, 26.7, 17.8 and 11.9% content (a geometric series with a factor of 1.5) of stock solution 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 produce 100 mg/L (nominal concentration). They were stirred for about 48 hours under closed system, and then a stock solution was prepared by taking out from the middle layer of the solution after settling for 1 hour. Test solution was prepared by diluting the stock solution with the dilution water appropriately.
(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 in test solution	GC analysis (at the start of the exposure, before and after the renewal (or at no surviving fish) and the end of the exposure)

Results

(1) Solubility of test item in dilution water (24±1°C) 19.7 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 2.00 to 10.0 mg/L

Before the renewal (or at no surviving fish) and at the end of the exposure 1.58 to 9.71 mg/L (69.3 to 98.8%)

(3) 96-hour LC_{50} (Median Lethal Concentration) 5.78 mg/L (95% confidence interval; 4.92 to 6.89 mg/L)

(4) The lowest concentration causing 100% mortality at 96 hours 9.87 mg/L

(5) The highest concentration causing 0% mortality at 96 hours 3.06 mg/L

[The values of (3), (4), (5) are based on geometric mean of the measured concentrations.]

1. Test item

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

1.1 Chemical name[*2]

2-(perfluorohexyl)ethanol

1.2 Chemical structure etc.[*2]

Structural formula

$$HOCH_2CH_2CF_2CF_2CF_2CF_2CF_2CF_3$$

Molecular formula	$C_8H_5F_{13}O$
Molecular weight	364.10
CAS Number	647-42-7

*2 Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number*2

Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	180804

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	78°C (14 mmHg)	
Density	1.678 g/cm^3 (20°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 March 16, 2007 were acclimated for 21 days (June 11 to July 2, 2007) using 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. The mortality was 0% during the 7 days before the start of the exposure. The test organism at the start of the exposure was 3-month-old fish. The test organisms were not treated with a medicament for external disinfection. The test organisms were fed on 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 values of the reference substance shown above are expressed as value converted into $CuSO_4$.

(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

This study was conducted with 5 exposure levels which were 60.0, 40.0, 26.7, 17.8 and 11.9% (a geometric series with a factor of 1.5) content of a stock solution prepared using the method shown in 3.5. The test concentrations and the factor were decided from the result of preliminary study. The results of preliminary study 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 for 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 [1.678 g/cm^3 (20°C)] for the preparation of test solution.

The test sample was added into Erlenmeyer flask filled with the dilution water using micro volumeter (Eppendorf Co., Ltd.) under the water surface to produce 100 mg/L (nominal concentration), and the flask was immediately sealed with a plug not to produce head space. Then, the solution was gently stirred by a magnetic stirrer for about 48 hours. After cease of the stirring, the solution was settled for 1 hour at 24±1°C, and then stock solution was prepared by taking out from the middle layer of the settled solution. The desired amount of the stock solution and the dilution water were mixed and stirred to prepare the test solution, and the prepared test solution was immediately divided into each test vessel and covered with a glass lid not to produce head space. The added amount of the stock solution for the preparation in each exposure level is shown in the next page.

Exposure level Content of stock solution (%)	Added amount of stock solution (mL/4L[*3])
Control	-
11.9	476
17.8	712
26.7	1068
40.0	1600
60.0	2400

*3 This contains sampling volume (about 100 mL)

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. The dead test organisms were removed immediately.

(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). For the exposure level where all fish were dead before the renewal, the observation was carried out at the point in time the death of all fish was confirmed.

(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. For the exposure level where all fish were dead before the renewal, the measurements were carried out at the point in time the death of all fish was confirmed. At the start of the exposure and after the renewal (at the preparation), another solution sampled from the container for the preparation was used for the measurement. Before the renewal and at the end of exposure, 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 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 solution was measured at the start of the exposure, before and after the renewal, and the end of the exposure. For the exposure level where all fish were dead before the renewal, the measurements were carried out at the point in time the death of all fish was confirmed. At the start of the exposure and after the renewal (at the preparation), the solution sampled from the container for the preparation was used for the measurement. Before the renewal and after the end of exposure, equal volume of the test solution was taken out from the middle layer of the test vessel in each test level (sampled from another vessel where all fish were dead in a vessel) and the mixture was used for the measurement. The concentration of the test item was analyzed by gas chromatography (GC). Analytical method and measured concentration of the test item 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}[*4]

The 24- and 48-hour LC_{50} values were estimated from Binomial test. The 72- and 96-hour LC_{50} values and the 95% confidence intervals were calculated by Probit analysis and the slope of the dose-response curve was also estimated.

The estimation of LC_{50} was performed based on the geometric mean of the measured concentration of the test solution during the exposure.

*4 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. Results and discussion

The content of the stock solution and the geometric mean of the measured concentration are shown in the following table.

Exposure level Content of stock solution (%)	Geometric mean of measured concentration (mg/L)
11.9	1.99
17.8	3.06
26.7	4.61
40.0	6.29
60.0	9.87

The following concentrations in this text are expressed using the geometric mean of the measured concentration.

4.1 Mortality

The lowest concentration causing 100% mortality within 96 hours was 9.87 mg/L. The highest concentration causing no mortality was 3.06 mg/L. Cumulative mortality at every time is shown in Table 1. Concentration-cumulative mortality curve is shown in Figure 1. Cumulative mortality in the control at the end of 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

No abnormal responses were observed in the control.

The following results of observation were based on the comparison with the control organisms. The observed abnormal responses during exposure were swimming at surface, loss of equilibrium, hemorrhage, lethargic, and reduced activity. 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 2.3 ± 0.14 cm

Body weight 0.10 ± 0.016 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. The appearance kept until before the renewal (or at the point in time of death of all fish).

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature during the exposure ranged from 6.2 to 8.6 mg/L, 7.1 to 7.9 and 23.5 to 24.0°C, respectively. Qualities 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*5 at the water temperature).

*5 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 solutions at the start of the exposure and after the renewal (at the preparation) ranged from 2.00 to 10.0 mg/L, and before the renewal (or at the point in time of death of all fish) and at the end of the exposure ranged from 1.58 to 9.71 mg/L which were 69.3 to 98.8% of the concentration at the preparation. The results of the measured concentrations of the test item are shown in Appendix 2.

4.5 LC_{50}

The 48-hour and 96-hour LC_{50} of 13F-EtOH to Medaka were 6.66 mg/L and 5.78 mg/L (95% confidence interval; 4.92 to 6.89 mg/L), respectively. The LC_{50}s at each observation time are shown in Table 4.

4.6 Discussion

This study was conducted to determine the LC_{50} of the test item to the test organisms below the solubility in the dilution water. The test was carried out under the condition of renewal of the test solution every day, since a slight reduction of the test item concentration in the test solution at 24 hours after the preparation was expected from the results of preliminary study. The measured concentrations of the test item maintained within 69.3 to 98.8% of the concentration at the preparation, the environmental conditions were also within the suitable range. Therefore, we evaluated that this study complied with the applied test guidelines.

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*6 (mg/L)	Cumulative mortality (%)				
	3 hours	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0	0
1.99	0	0	0	0	0
3.06	0	0	0	0	0
4.61	0	0	0	10	30
6.29	0	30	40	50	50
9.87	0	100	100	100	100

*6 Geometric mean of measured concentration (expressed as measured concentration in the following)

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
1.99	0/10	N	0/10	N	0/10	N	0/10	N	0/10	N
3.06	0/10	N	0/10	N	0/10	N	0/10	N	0/10	N
4.61	0/10	N	0/10	N	1/10	AS(1)	1/9	PLE(1) RA(1)	1/7	AS(1) RA(1)
6.29	0/10	N	2/7	HEM(1) PLE(1) RA(1)	2/6	AS(1) PLE(1) RA(1)	2/5	PLE(2)	3/5	AS(1) LETH(1) PLE(1) RA(2)
9.87	4/10	CLE(3) HEM(2) LETH(1) RA(3)	-	-	-	-	-	-	-	-

N : Normal (No abnormal response)

Values in parentheses express number of affected fish.

- shows that all fish have died.

Abbreviation of symptoms

AS: At the surface

CLE: Complete loss of equilibrium

HEM: Hemorrhage

LETH: Lethargic

PLE: Partial loss of equilibrium

RA: Reduced activity

Table 3-1 Dissolved oxygen concentration of test solutions

Measured concentration (mg/L)	0 hours	24 hours		48 hours		72 hours		96 hours
	At start	Before renewal	After renewal	Before renewal	After renewal	Before renewal	After renewal	At end
Control	7.8	6.2	7.8	6.9	8.2	6.8	8.1	6.8
1.99	8.5	6.5	8.4	7.2	8.5	6.9	8.4	6.5
3.06	8.5	6.6	8.3	7.4	8.6	6.9	8.5	6.7
4.61	8.5	6.6	8.3	7.2	8.5	7.0	8.4	6.8
6.29	8.5	6.5	8.4	7.6	8.3	7.6	8.5	6.6
9.87	8.2	7.2*	-	-	-	-	-	-

Unit : mg/L

- shows that all fish have died.

* shows that the value was measured when the death of all fish was confirmed.

Table 3-2 pH of test solutions

Measured concentration (mg/L)	0 hours	24 hours		48 hours		72 hours		96 hours
	At start	Before renewal	After renewal	Before renewal	After renewal	Before renewal	After renewal	At end
Control	7.7	7.3	7.9	7.4	7.6	7.3	7.6	7.3
1.99	7.8	7.3	7.8	7.2	7.7	7.2	7.6	7.2
3.06	7.8	7.3	7.8	7.3	7.7	7.2	7.6	7.1
4.61	7.8	7.3	7.8	7.3	7.7	7.2	7.6	7.1
6.29	7.8	7.3	7.8	7.4	7.7	7.3	7.7	7.1
9.87	7.8	7.4*	-	-	-	-	-	-

- shows that all fish have died.

* shows that the value was measured when the death of all fish was confirmed.

Table 3-3 Temperature of test solutions

Measured concentration (mg/L)	0 hours	24 hours		48 hours		72 hours		96 hours
	At start	Before renewal	After renewal	Before renewal	After renewal	Before renewal	After renewal	At end
Control	23.9	23.8	23.7	23.5	23.8	23.5	23.7	23.9
1.99	23.8	23.8	23.7	23.5	23.8	23.5	23.8	23.9
3.06	23.8	23.8	23.7	23.5	23.8	23.5	23.8	23.9
4.61	23.8	23.8	23.7	23.6	23.8	23.5	23.9	23.9
6.29	24.0	24.0	23.8	23.6	23.8	23.5	23.9	23.9
9.87	24.0	24.0*	-	-	-	-	-	-

Unit: °C

- shows that all fish have died.

* shows that the value was measured when the death of all fish was confirmed.

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 hours	6.97		Binomial test
48 hours	6.66		Binomial test
72 hours	6.16	5.37 to 7.31 (11.2)	Probit analysis
96 hours	5.78	4.92 to 6.89 (8.18)	Probit analysis

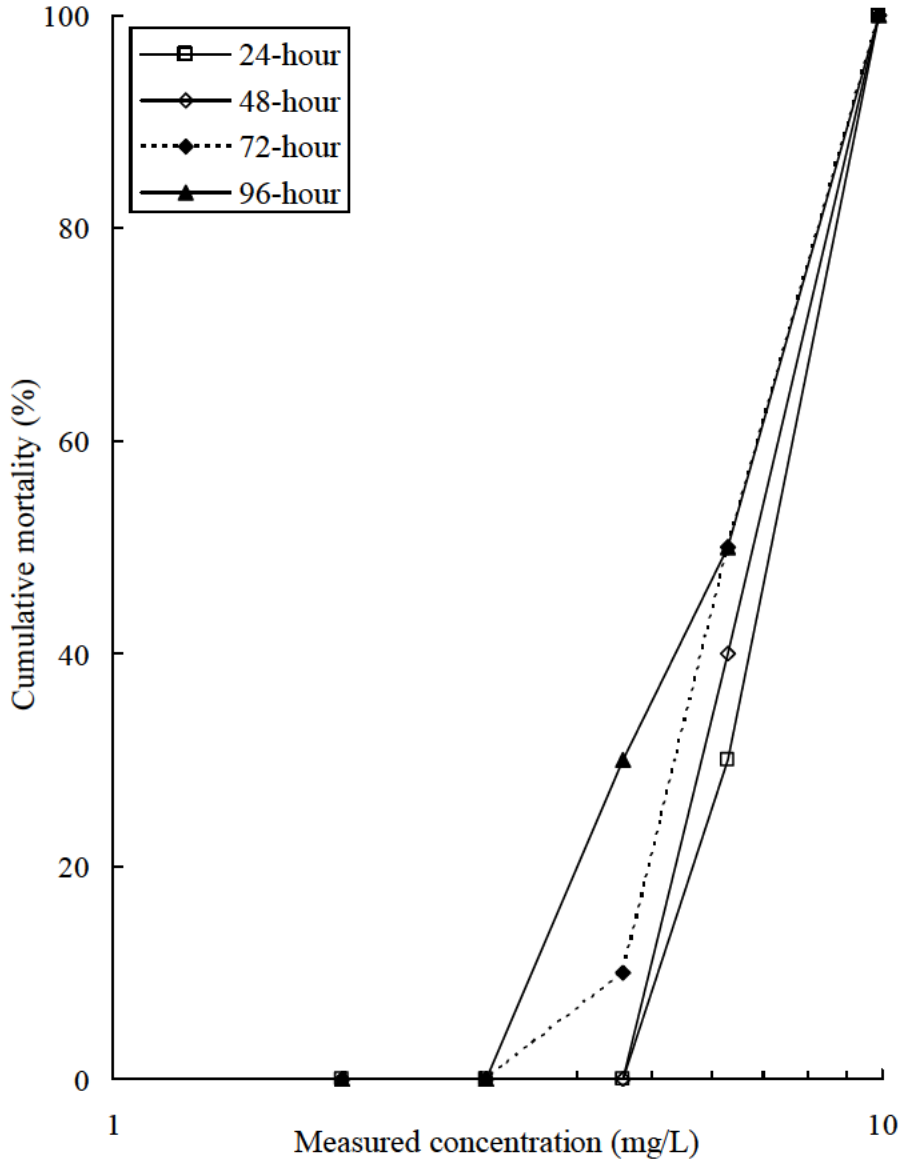


Figure 1 Concentration-cumulative mortality curve.

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

1. Pretreatment of test solution

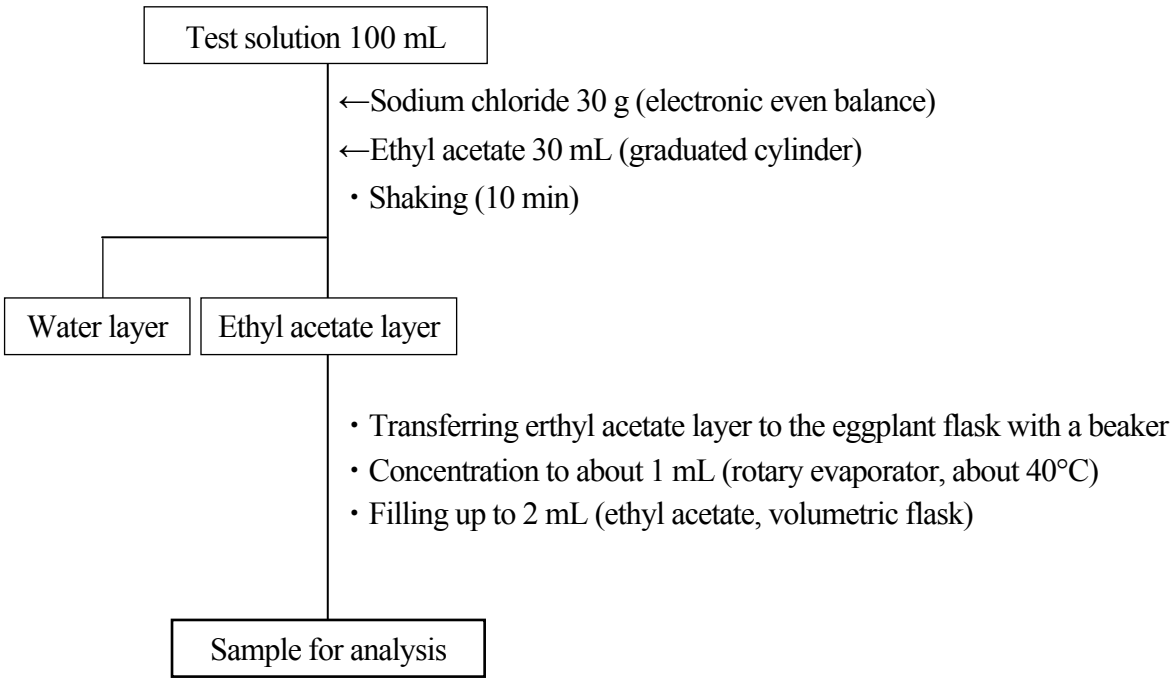
① 26.7, 40.0 and 60.0%

The test solutions sampled were used as the samples for analysis after appropriate dilution to produce methanol / dechlorinated tap water (1/1 v/v).

② Control, 11.9 and 17.8%

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

Flow scheme



2. 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. The concentrations of the test item 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 exceeded the range of the calibration curve were diluted to fall within the range and analyzed. Some chromatograms obtained are shown in Appendix 3.

① Analytical conditions(26.7, 40.0 and 60.0%)

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) $\xrightarrow{①}$ 110°C(0 min) $\xrightarrow{②}$ 240°C(2 min)
Temp. rate	①10°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

② Analytical conditions(Control, 11.9 and 17.8%)

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

3. Preparation of standard solution

The standard solutions to determine the concentrations of the test item 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.

① 26.7, 40.0 and 60.0%

The test sample of 100.2 mg was precisely weighed with an electronic balance and dissolved in methanol to obtain 1000 mg/L solution of the test item. The test item solution was diluted with methanol / dechlorinated tap water (1/1 v/v) to prepare 50.0 mg/L of test item solution. The solution was diluted with methanol / dechlorinated tap water (1/1 v/v) to prepare 5.00 mg/L of standard solution.

② Control, 11.9 and 17.8%

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 test item solution was diluted with ethyl acetate to prepare 5.00 mg/L of standard solution.

4. Calibration curve

① 26.7, 40.0 and 60.0%

The standard solutions of 0.500, 2.50, 5.00, 10.0 and 20.0 mg/L were prepared by the same procedure as described in section 3(Preparation of standard solution ①). These solutions were analyzed according to the quantitative analytical conditions described in section 2 ①. The calibration curve was drawn from the relationship between the concentrations of standard solution and the peak area on the chromatogram, and the determination was confirmed. The calibration curve is shown in Appendix 3.

② Control, 11.9 and 17.8%

The standard solutions of 0.500, 2.50, 5.00, 10.0 and 20.0 mg/L were prepared by the same procedure as described in section 3 (Preparation of standard solution ②). These solutions were analyzed according to the quantitative analytical conditions described in section 2 ②. The calibration curve was drawn from the relationship between the concentrations of standard solution 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 (0.500 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of the test item in the test solution was 0.0124 mg/L in consideration of pretreatment.

5. Recovery test and blank test

5.1 Method

The recovery test was conducted by adding the test item solution (prepared with acetone) to dilution water according to pretreatment of test solution described in section 1(Pretreatment of test solution ②). The blank test was also conducted using dilution water (added acetone) without the test item 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 10.0 μg

5.2 Result

As a result of analysis by the method of section 5.1, no peak of the test item appeared in the chromatogram of the blank test. Two individual recovery rates and their average on the analytical procedure are shown below. The average of recovery rate was used as correction factor, for the determination of the test item concentrations in the test solutions.

Recovery rate of the test item for pretreatment

81.7%, 80.1% average 80.9%

6. Results of measurement

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

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

Stock solution (%)	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.	
11.9	2.25	1.94 (86.1)	2.34	1.91 (81.6)	2.40	1.66 (69.3)	2.00	1.58 (79.1)	1.99
17.8	3.55	2.94 (82.7)	3.45	3.06 (88.8)	3.61	2.55 (70.6)	3.17	2.39 (75.4)	3.06
26.7	5.18	4.60 (88.8)	5.23	4.46 (85.2)	4.93	4.31 (87.4)	4.18	4.10 (98.2)	4.61
40.0	7.02	6.23 (88.8)	6.54	5.64 (86.3)	6.47	6.28 (97.2)	6.16	6.09 (98.8)	6.29
60.0	10.0	9.71* (96.8)	-	-	-	-	-	-	9.87

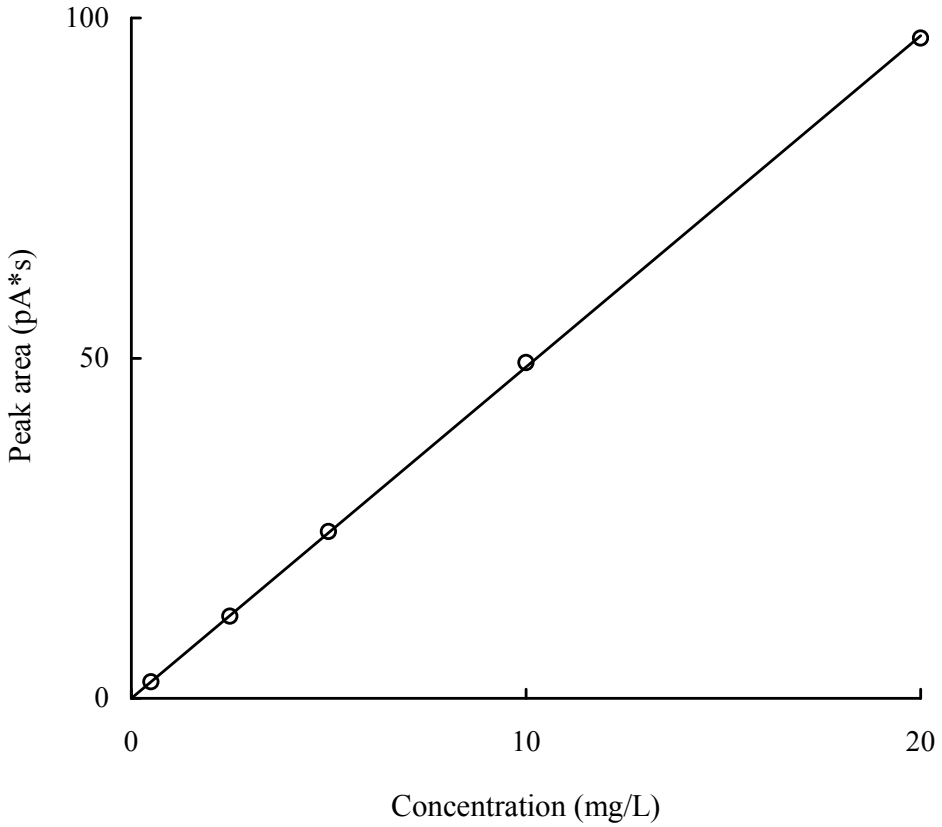
- : It shows no measurement because all test organisms died.

* It indicates the measured value at the time that confirmed all test organisms dead.

n.d. : < 0.0124 mg/L

Appendix 3

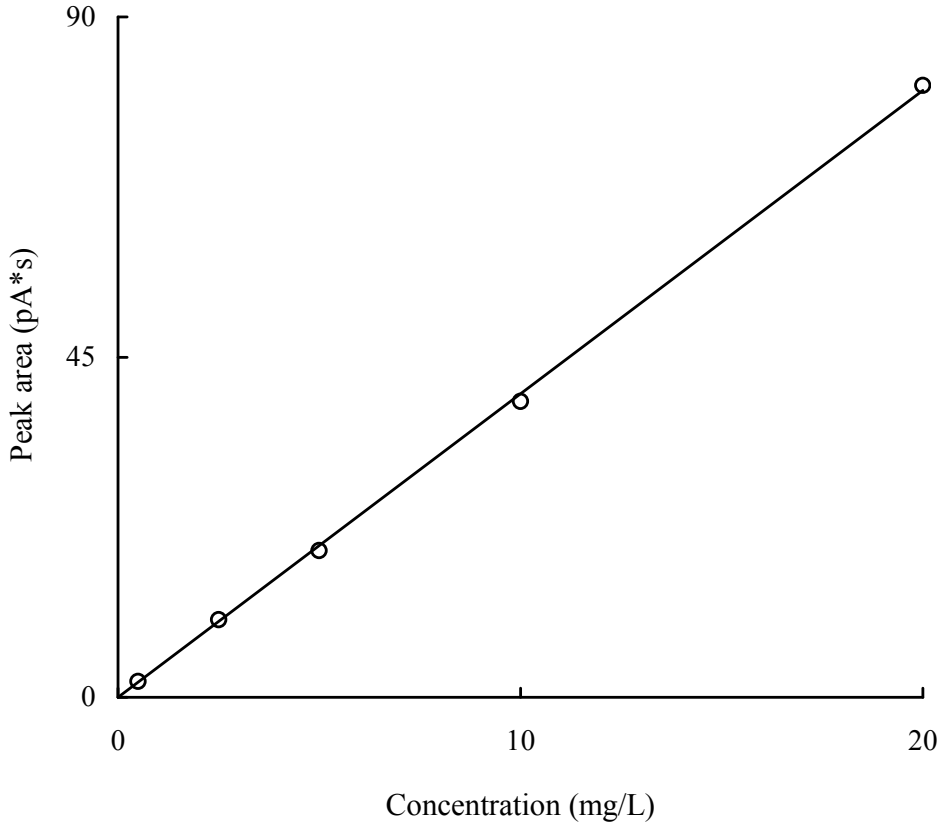
Calibration curve and chromatogram



y = 4.87x
r = 1.00

Concentration (mg/L)	Peak area (pA*s)
0.500	2.415
2.50	12.068
5.00	24.514
10.0	49.334
20.0	97.037

Appendix figure 3-1-1 Calibration curve of 13F-EtOH for analysis by GC (Control, 11.9 and 17.8%).



y = 4.01x
r = 1.00

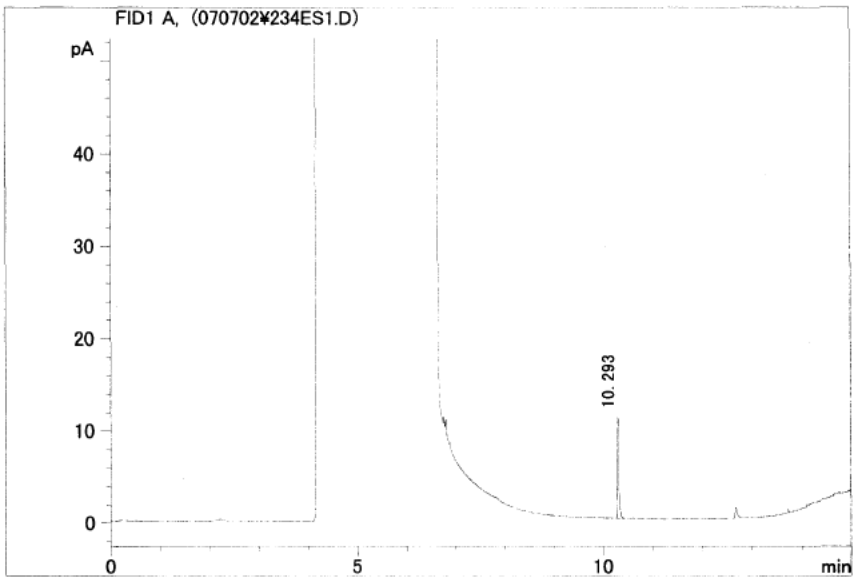
Concentration (mg/L)	Peak area (pA*s)
0.500	2.122
2.50	10.278
5.00	19.391
10.0	39.164
20.0	80.927

Appendix figure 3-1-2 Calibration curve of 13F-EtOH for analysis by GC(26.7, 40.0 and 60.0%).

Standard solution 5.00 mg/L

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070702¥234ES1.D
サンプル名 : std 5.00mg/L



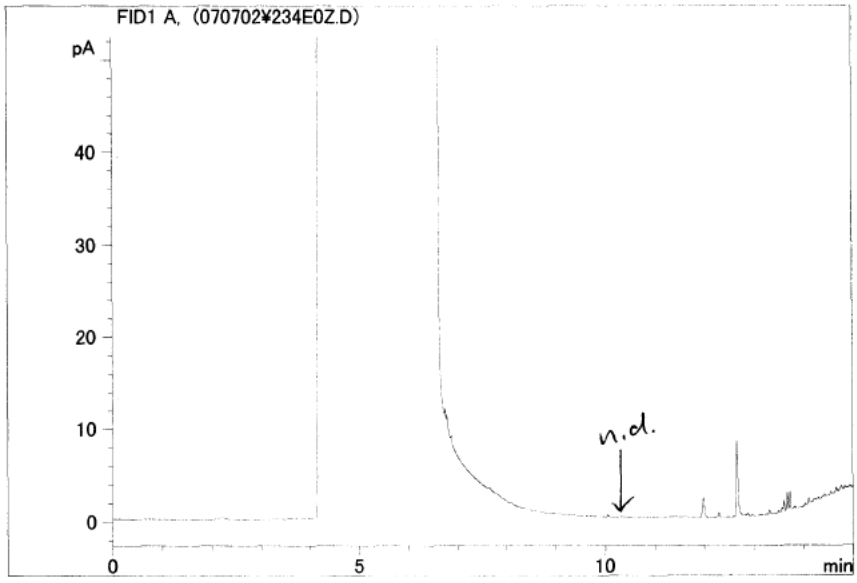
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	24.422	10.975	10.293

Appendix figure 3-2-1 GC chromatogram of standard solution at start of exposure (Control, 11.9 and 17.8%).

Control

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070702¥234E0Z.D
サンプル名 : Cont(*50)Y0h



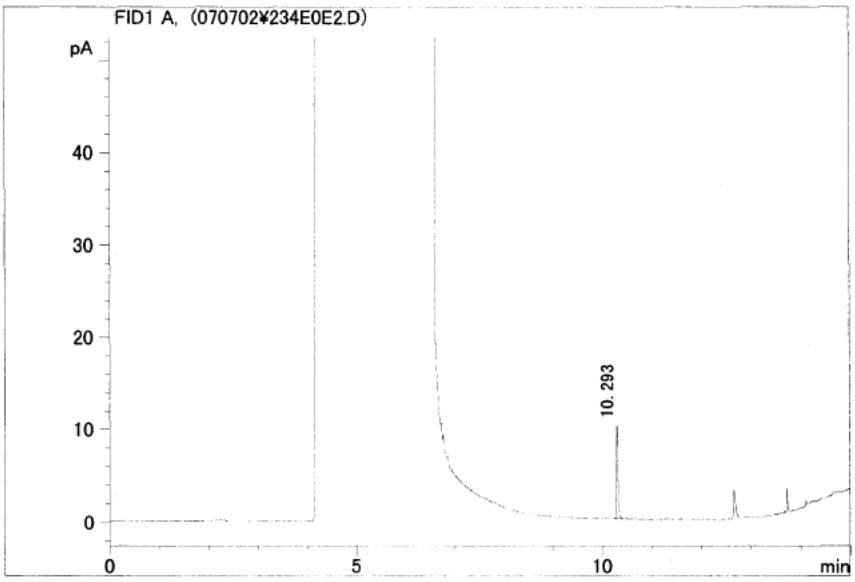
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

11.9% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070702¥234E0E2.D
サンプル名 : 11.9%(*50D20)Y0h



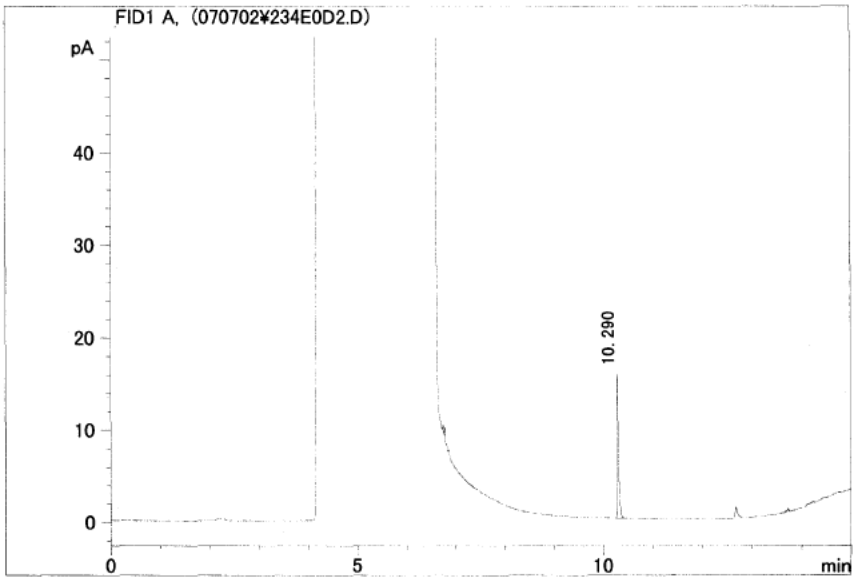
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	22.262	9.913	10.293

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

17.8% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070702¥234E0D2.D
サンプル名 : 17.8%(*50D20)Y0h



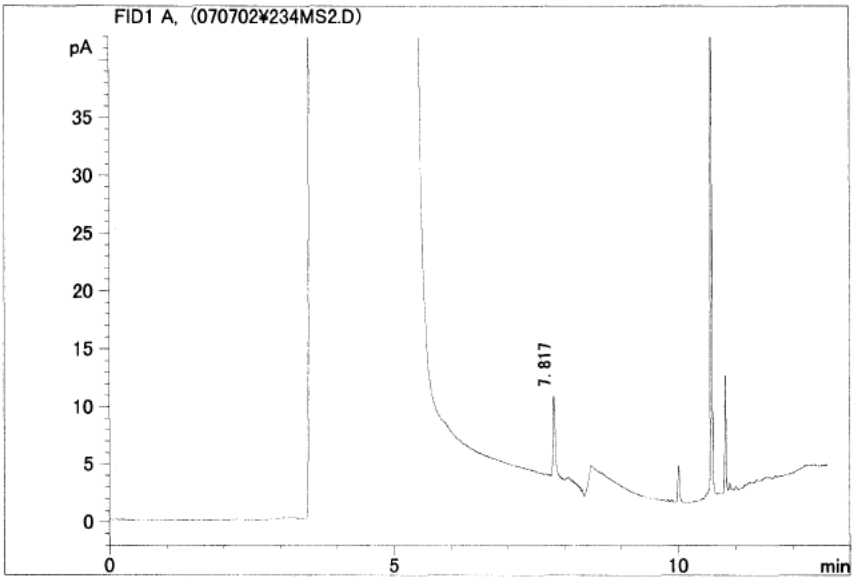
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	35.053	15.588	10.290

Appendix figure 3-2-4 GC chromatogram of test solution at start of exposure.

Standard solution 5.00 mg/L

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070702¥234MS2.D
サンプル名 : std 5.00mg/L



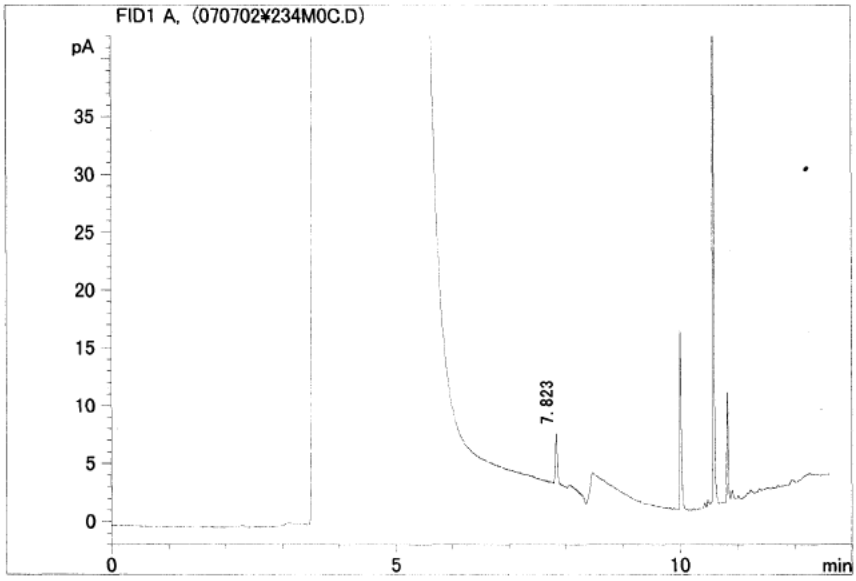
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.163	7.057	7.817

Appendix figure 3-2-5 GC chromatogram of standard solution at start of exposure (26.7, 40.0 and 60.0%).

26.7% (Stock solution)

Study No. 94234

測定オペレータ　　:
メソッド名　　: C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070702¥234M0C.D
サンプル名　　: 26.7%~~(+80)~~Y0h　　入力ミスにより訂正 2007.7.3 安達
(D2)



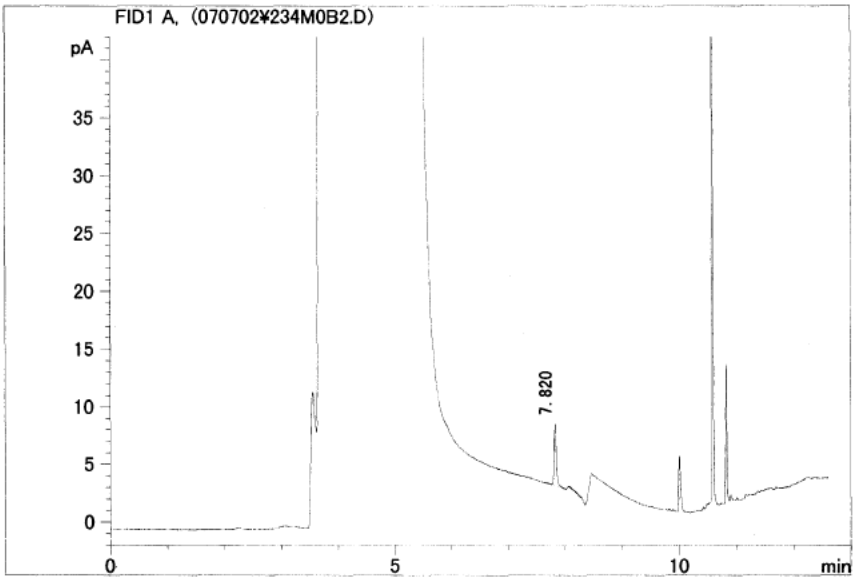
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	9.919	4.286	7.823

Appendix figure 3-2-6　GC chromatogram of test solution at start of exposure.

40.0% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070702¥234M0B2.D
サンプル名 : 40.0%(D2)Y0h



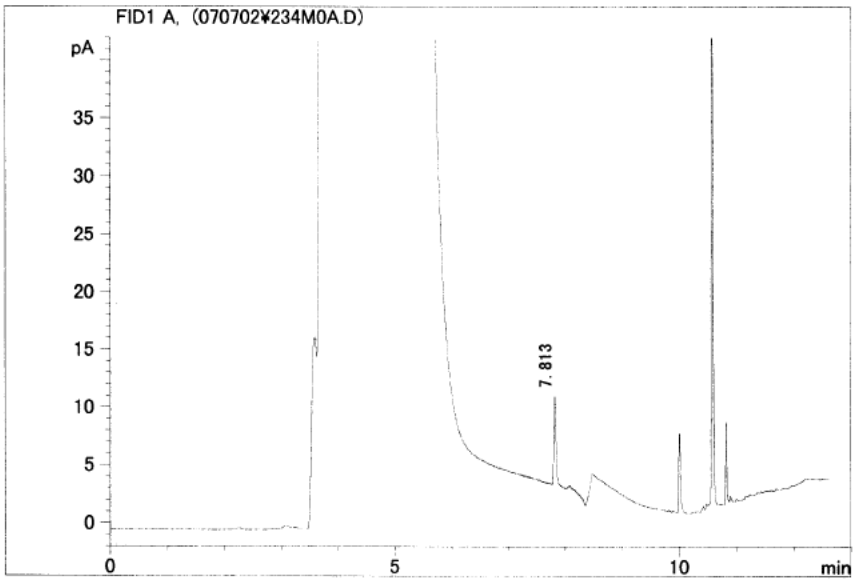
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	13.455	5.305	7.820

Appendix figure 3-2-7 GC chromatogram of test solution at start of exposure.

60.0% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070702¥234M0A.D
サンプル名 : 60.0%(D2)Y0h



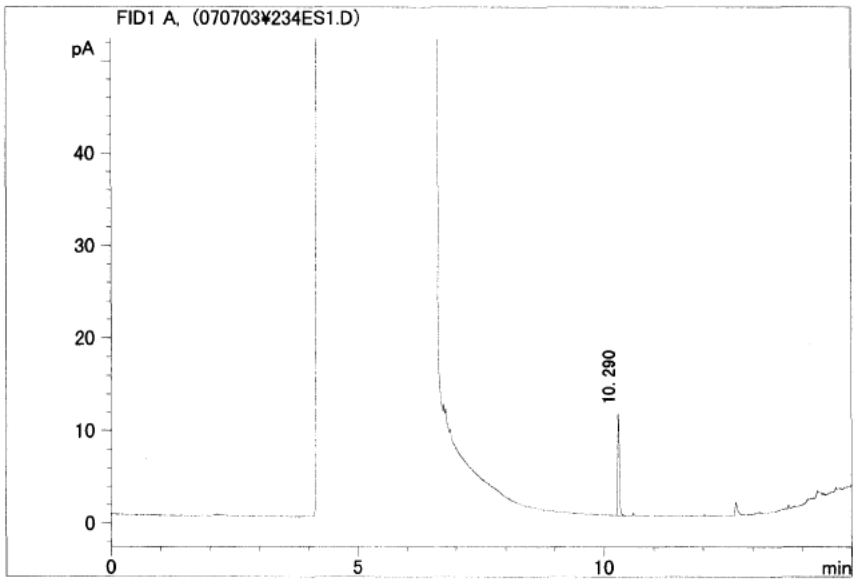
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.224	7.555	7.813

Appendix figure 3-2-8 GC chromatogram of test solution at start of exposure.

Standard solution 5.00 mg/L

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070703¥234ES1.D
サンプル名 : std 5.00mg/L



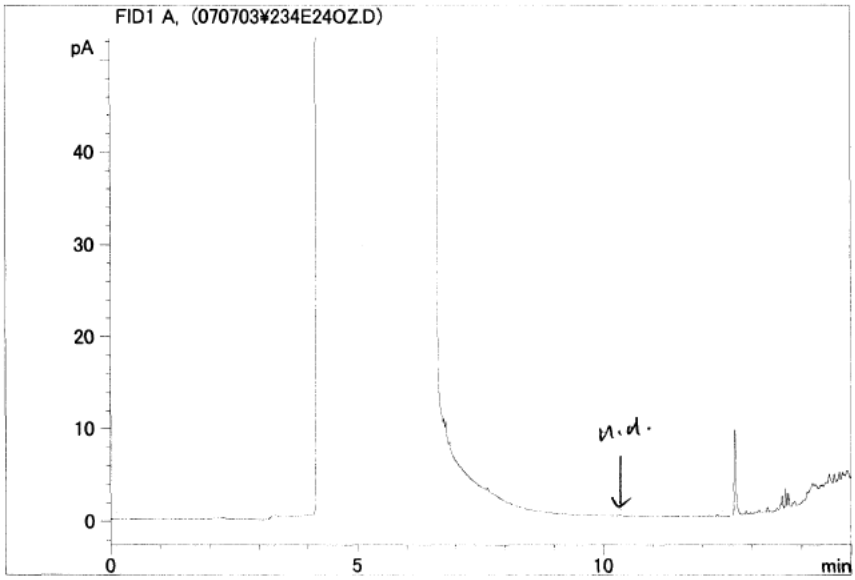
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	25.195	11.080	10.290

Appendix figure 3-3-1 GC chromatogram of standard solution before renewal at 24 hours (Control, 11.9 and 17.8%).

Control

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070703¥234E24OZ.D
サンプル名 : Cont(*50)Y24h01d



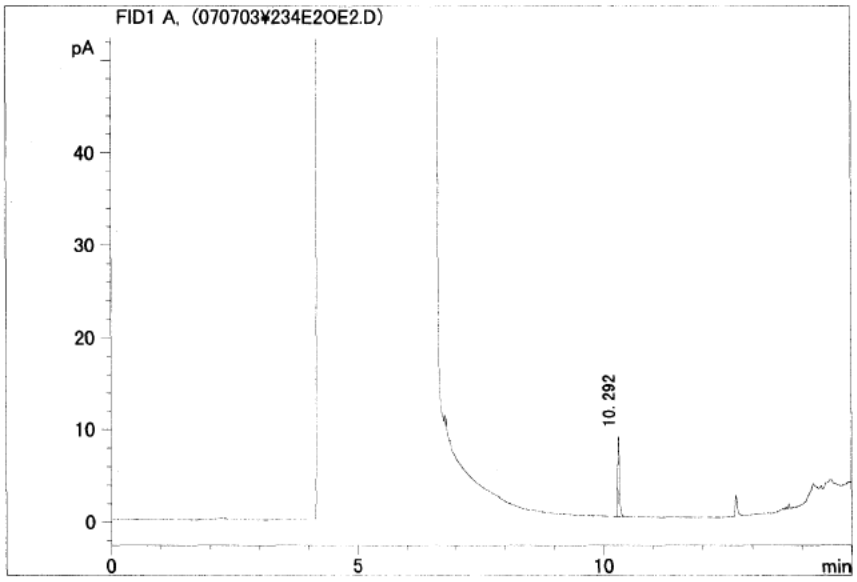
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

11.9% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070703¥234E20E2.D
サンプル名 : 11.9%(*50D20)240



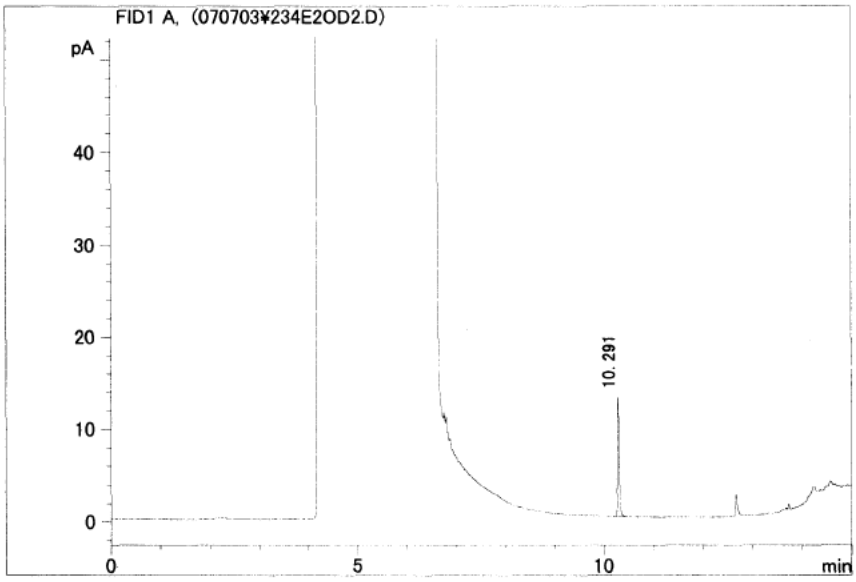
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.778	8.654	10.292

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

17.8% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070703¥234E20D2.D
サンプル名 : 17.8%(*50D20)240



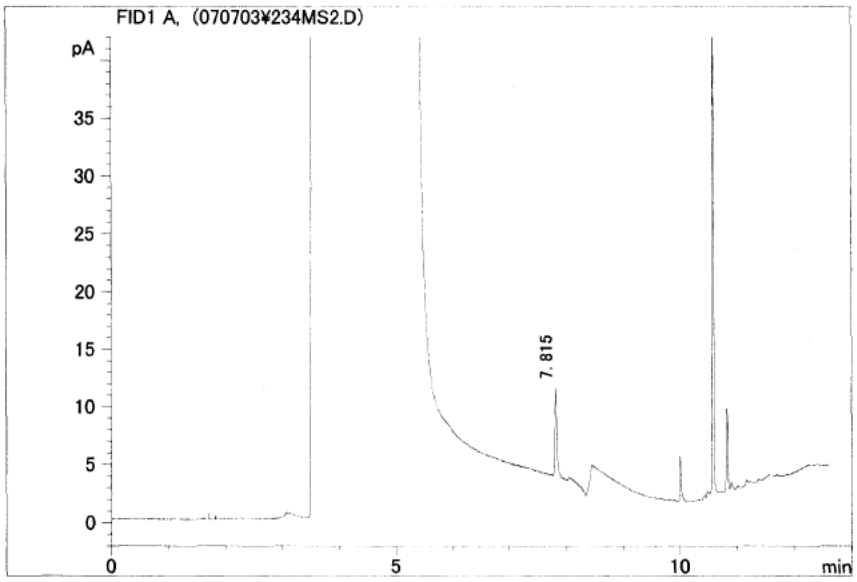
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	29.919	12.887	10.291

Appendix figure 3-3-4 GC chromatogram of test solution before renewal at 24 hours

Standard solution 5.00 mg/L

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名: C:¥HPCHEM¥1¥DATA¥070703¥234MS2.D
サンプル名 : std 5.00mg/L



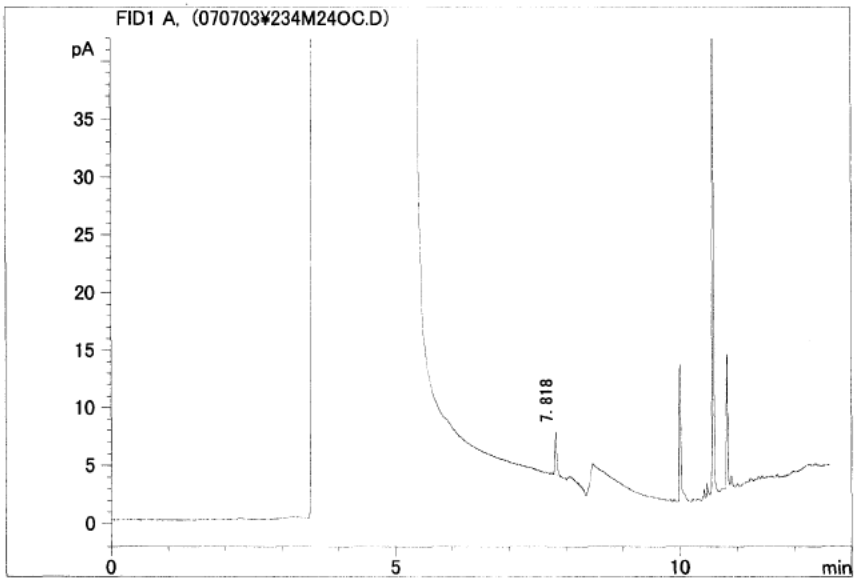
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	19.175	7.608	7.815

Appendix figure 3-3-5 GC chromatogram of standard solution before renewal at 24 hours (26.7, 40.0 and 60.0%).

26.7% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070703¥234M24OC.D
サンプル名 : 26.7%(D2)Y24h01d



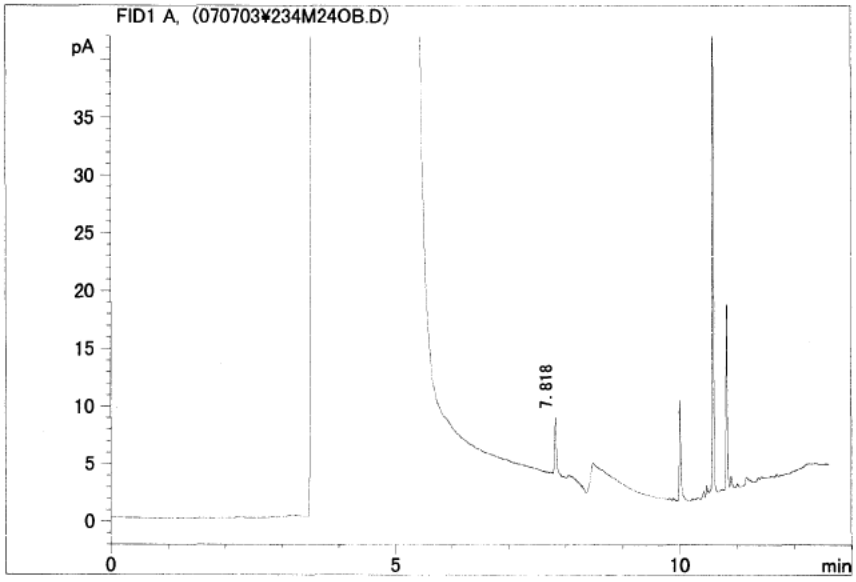
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	8.813	3.702	7.818

Appendix figure 3-3-6 GC chromatogram of test solution before renewal at 24 hours.

40.0% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070703¥234M240B.D
サンプル名 : 40.0%(D2)Y24h01d



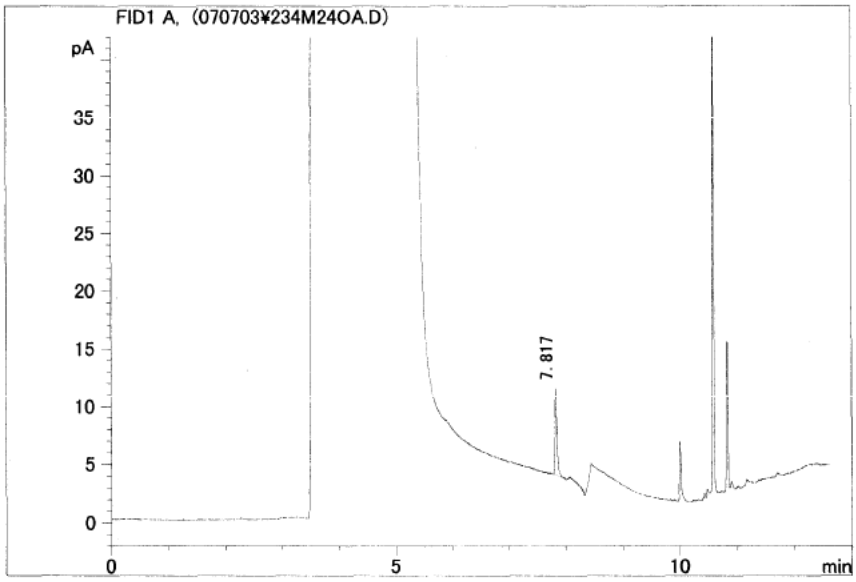
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	11.951	4.914	7.818

Appendix figure 3-3-7 GC chromatogram of test solution before renewal at 24 hours.

60.0% (Stock solution)

Study No. 94234

測定オペレータ :
メソッド名 : C:¥HPCHEM¥1¥METHODS¥94232M.M
生データファイル名 : C:¥HPCHEM¥1¥DATA¥070703¥234M240A.D
サンプル名 : 60.0%(D2)Y24h01d



#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	18.612	7.430	7.817

Appendix figure 3-3-8 GC chromatogram of test solution 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 and 48hours 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 : Twice (after the mixture was stirred for 24 and 48 hours)

(3) Dilution water : Dechlorinated tap water

(4) Repetition : 24 hours n=3 (Sample-1, Sample-2 and Sample-3)
48 hours n=3 (Sample-4, Sample-5 and Sample-6)

4.3 Test procedures

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

*1 The additive amount (35.8 μL) was caluculated from the density of the test item (1.678 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 or 48 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. In addition, three analyses were conducted from the each sample.

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 solutions were used as the samples for analysis after appropriate dilution to produce methanol / dechlorinated tap water (1/1 v/v).

(2) Method for analysis

See Appendix 2 2. Method of analysis ①.

4.5 Preparation of standard solution

See Appendix 2 3. Preparation of standard solution ①.

4.6 Calibration curve

See Appendix 2 4. Calibration curve ①.

5. Results

Measured solubility of the test item after 48 hours stirring was higher than that of after 24 hours stirring. Therefore, value of after 48 hours stirring was adopted to the solubility in dilution water. The solubility of the test item to medium was 19.7 mg/L. The results of analyses are shown below.

Appendix table 4-1 Value measured after stirring for 24 hours

Sample name		Measured value (mg/L)	Average value *2 (mg/L)	Total average value *2 (mg/L)
Sample-1	a	12.4	12.9	13.3
	b	14.1		
	c	12.3		
Sample-2	a	13.0	12.6	
	b	12.2		
	c	12.6		
Sample-3	a	15.5	14.5	
	b	13.7		
	c	14.2		

Appendix table 4-2 Value measured after stirring for 48 hours

Sample name		Measured value (mg/L)	Average value *2 (mg/L)	Total average value *2 (mg/L)
Sample-4	a	20.2	20.5	19.7
	b	21.1		
	c	20.2		
Sample-5	a	18.5	19.6	
	b	19.9		
	c	20.2		
Sample-6	a	19.7	19.0	
	b	19.0		
	c	18.5		

*2 Arithmetic mean value

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 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. In addition, three repetitions per container by sampling of middle layer were analyzed to check the variation of the test item concentration. The concentration of the test item in the collected sample was analyzed by gas chromatography (GC) after the pretreatment.

(2) Result

Value measured after stirring for 24 hours

Sample name		Measured value(mg/L)	Arithmetic mean value (mg/L)
Sample-1	①	18.8	19.1
	②	18.0	
	③	20.6	
Sample-2	①	14.9	14.1
	②	13.5	
	③	13.8	

Value measured after stirring for 48 hours

Sample name		Measured value(mg/L)	Arithmetic mean value (mg/L)
Sample-3	①	18.4	19.5
	②	19.4	
	③	20.8	
Sample-4	①	23.0	23.2
	②	22.0	
	③	24.4	

Solubility of test item in dilution water was around 13 to 25 mg/L

2) Summary of preliminary study for measurement of solubility

By the results of preliminary study 1 for measurement of solubility, the solubility of the test item in dilution water was around 13 to 25 mg/L. The test solutions were gently stirred. And then, the middle layer was sampled after settling for about 1 hour for removal of insoluble substance, because the centrifugation and filtration with a membrane filter caused the decrease of test item concentration. By the results of three repetitions per sample, the test concentrations were nearly same value. So, it was considered that the removal of insoluble substance by the method stated above was successful.

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 solution was gently stirred by magnetic stirrer, the solution was settled for 1 hour after cease of stirring, and the test solution was prepared by taking out from the middle layer of the settled solution.

2. Effect on test organism

1) Preliminary study

(1) Method

The test sample was added into Erlenmeyer flask filled with the dilution water using micro volumeter (Eppendorf Co., Ltd.) under the water surface to produce 100 mg/L (nominal concentration), and the flask was immediately sealed with a plug to produce no headspace. Then the solution was gently stirred with a magnetic stirrer for about 48 hours. After cease of stirring, the solution was settled for about 1 hour, and then a stock solution was prepared by taking out from the middle layer of the settled solution. Test solution was prepared by mixing the desired amount of the stock solution and the dilution water. The test organisms were exposed to the test solution, and the test solution was immediately closed by covering with a glass lid to produce no headspace. Afterward, the effect on the organisms was observed, and the maintenance of the test item concentration was also investigated.

(2) Result

Content of stock solution (%)	Left column : Cumulative mortality (%) Right column : Existence of abnormal response (abnormalities : *, no abnormalities : N)									
	3 hours		24 hours		48 hours		72 hours		96 hours	
Control	0	N	0	N	0	N	0	N	0	N
10.0	0	N	0	N	0	N	0	N	0	N
30.0	0	N	0	N	0	*	0	*	0	*
60.0	0	N	100	-	100	-	100	-	100	-

- shows that all fish have died.

Exposure system: semi-static (renewal once a day)

Number of organisms/volume of test solution: 5 fish/about 3.3 L

Aeration: none

<Measured concentration of test item in test solution>

Content of stock solution (%)	Measured concentration (mg/L) (percentage of measured concentration at start)	
	At the start	After 24 hours
Control	n.d.	n.d.
10.0	1.82	1.59 (87.0)
60.0	11.7	10.6 (90.7)

n.d.<1.00 mg/L

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

Since the test solution prepared with a stock solution of the saturated concentration, which was prepared by mixing and stirring the test sample and the dilution water to produce 100 mg/L, affected the test organisms in mortality, a definitive study planed to conduct with 5 exposure levels, and also using vessel with closed system without headspace and semi-static regime of renewal every 24 hours, taking into account the maintenance of the test item concentration in the test solution.

3. Operation of definitive study

1) Measurement of solubility of test item in dilution water

From the result of the preliminary study, the measurement of the solubility was performed using the solution taken out from the middle layer of the solution which was prepared by mixing the test sample and the dilution water to produce about 100 mg/L and stirred gently for 24 and 48 hours under the condition of 24±1°C and closed system. For removal of insoluble substance, the procedure of centrifugation or filtration was not used, but it of settling for about 1 hour after cease of stirring and then taking out from the middle layer of the settled solution was used as a method to remove as much as possible. The measurement of the test item concentration was employed for this test solution.

2) Definitive study

A definitive study was conducted with 5 exposure levels of which content of a stock solution, prepared by taking out from the middle layer of a solution with saturated concentration (nominal concentration; 100 mg/L) prepared by mixing the test sample and the dilution water for about 48 hours, were 60.0, 40.0, 26.7, 17.8 and 11.9% (a geometric series with a factor 1.5). The test was also performed with renewal of the test solution once a day, no aeration during the exposure, and closed system. As to preparation of the test solution, the test sample was added into Erlenmeyer flask filled with the dilution water using micro volumeter (Eppendorf Co., Ltd.) under the water surface to produce 100 mg/L (nominal concentration), and the flask was immediately sealed with a plug not to produce head space. Then, the solution was gently stirred by a magnetic stirrer for about 48 hours. After cease of the stirring, the solution was settled for 1 hour at 24±1°C and then a stock solution was prepared by taking out from the middle layer of the settled solution. The desired amount of the stock solution and the dilution water were mixed and stirred to prepare the test solution. The test item concentration in the test solution was measured for all exposure levels at the start of the exposure, before and after the renewal, and the end of the exposure.