Receipt number	662-06-E-4233
Study number	94233

FINAL REPORT

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

July 26, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

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

Study number 94233

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.

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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	July 6, 2007	July 6, 2007
Study plan	July 6, 2007	July 6, 2007
Amendment of study plan	July 25, 2007	July 25, 2007
Measurement of solubility	July 11, 2007	July 13, 2007
	July 13, 2007	July 13, 2007
Start of the exposure and after the exposure	July 9, 2007	July 12, 2007
	July 10, 2007	July 12, 2007
	July 12, 2007	July 12, 2007
Raw data and final report draft	July 25, 2007	July 26, 2007
Final report	July 26, 2007	July 26, 2007

Date July 26, 2007

Head of Quality Assurance Unit Signed in original

CONTENTS

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

Applied GLP

This study was conducted in compliance with the following GLP principles:

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

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

Dates

Study initiation date	July 6, 2007
Experimental starting date	July 10, 2007
Solubility study starting date	July 11, 2007
Bioassay starting date	July 10, 2007
Experimental completion date	July 13, 2007
Solubility study completion date	July 13, 2007
Bioassay completion date	July 12, 2007
Study completion date	July 26, 2007

Storage of test item, raw data, etc.

(1) Test item

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

*1 It will be stored as the common sample for storage of these studies (Study Nos. 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 48-hour Acute Immobilization Study of 13F-EtOH with *Daphnia magna*

Test conditions

(1)	Test item	13F-EtOH
(2)	Test organism	*Daphnia magna*
(3)	Exposure duration	48 hours
(4)	Test concentration	Five concentrations of content of stock solution, 100, 55.6, 30.9, 17.1 and 9.53% (a geometric series with a factor of 1.8), and control
(5)	The number of organisms	Twenty daphnids/test level (five daphnids/test vessel)
(6)	Dilution water	Dechlorinated tap water
(7)	Type of test	Semi-static regime (renewal at 24 hours after) with closed system
(8)	Preparation of test solution	The test sample and dilution water were mixed to prepare 100 mg/L (nominal concentration), and they were stirred under closed system for approximately 48 hours. After settlement for approximately 1 hour, stock solution was prepared by taking out from the middle layer. Test solution was prepared by appropriately diluting the stock solution with dilution water.
(9)	Replicate	Four replicates/test level
(10)	Volume of test solution	Approximately 1000 mL/test level (approximately 250 mL/test vessel)
(11)	Temperature of test solutions	20±1℃
(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, and the end of the exposure)

Results

(1) Solubility in dilution water (20±1℃) 18.0 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 1.30 to 15.5 mg/L

Before the renewal and at the end of the exposure 1.31 to 13.5 mg/L (77.3 to 102%)

(3) 48-hour EC_{50} (Median Effective Concentration) 8.20 mg/L (95% confidence interval; 7.16 to 9.46 mg/L)

(4) Minimum concentration causing 100 per cent immobility during 48 hours 14.1 mg/L

(5) Maximum concentration causing no immobility during 48 hours 1.33 mg/L

[The concentrations shown in (3), (4) and (5) are based on a geometric mean of the measured concentrations.]

1. Test item

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

1.1 Name[*2]

2-(perfluorohexyl)ethanol

1.2 Structural formula 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 component 0.2%

2.3 Confirmation of test item supplied by the sponsor

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
	Dimethyl sulfoxide	Soluble (freely miscible)
	Acetone	Soluble (freely miscible)

*2 Information supplied by the sponsor

2.5 Storage condition and confirmation of stability at storage condition

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

3. Test materials and methods

3.1 Test organism

(1) Species

Daphnia magna (Clone A)

(2) Reason for selection of species

Species recommended in the test guidelines

(3) Source

Young daphnids produced by parents which were cultured in the Kurume Laboratory were used. Daphnids [*Daphnia magna* (Clone A)] originally came from the University of Sheffield (Address: Sheffield S10 2UQ, United Kingdom). The parents to obtain young daphnids were bred in the same quality of water (dechlorinated tap water), water temperature (20±1℃), photoperiod (16-hour light/8-hour dark) as used in the test. Parents used for the test were same lot and bred for more than 14 days, and their age and survival rate were 14-day old and 100%, respectively. *Chlorella vulgaris* of 0.1 to 0.2 mgC/day per *daphnia* was fed to the parents once a day. A 48-hour acute immobilization test of $K_2Cr_2O_7$ (Reagent grade, Wako Pure Chemical Industries, Ltd.) with the test organisms was conducted (on June 26 to 28, 2007) to confirm the reproducibility of the test conditions. The 48-hour EC_{50} of $K_2Cr_2O_7$ was 0.296 mg/L. This value was within the normal range in this laboratory (mean ± 2S.D.: 0.124 to 0.350 mg/L) [mean ± S.D.: 0.237± 0.057 mg/L (n=59)].

(4) Selection of young daphnids

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

(5) Allocation to the test groups

Test organisms were placed at random to each test vessel.

3.2 Dilution water

Dechlorinated tap water, aerated sufficiently and temperature-controlled, was used. Some chemical characteristics of the dilution water measured regularly are listed in Appendix 1.

3.3 Test apparatus and equipment

(1) Test apparatus

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

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

(2) Test equipment

Water bath : Plastic tank

Warming/cooling unit (Type HCA250, Sato craft)

3.4 Test conditions

(1) Conditions of exposure

(a) Type of test

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

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

(b) Exposure duration

48 hours

(c) Test concentration

The present study was conducted with 5 exposure levels, 100, 55.6, 30.9, 17.1 and 9.53% (a geometric series with a factor of 1.8), of content of stock solution prepared by the procedure described in 3.5. The exposure levels and the factor were decided by the results of preliminary studies. The results of the preliminary studies are shown in Additional data.

(d) Control

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

(e) Replicates

Four replicates/test level

(f) The number of organisms

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

(g) Volume of test solution

Approximately 1000 mL/test level (approximately 250 mL/test vessel)

(2) Conditions of test environment

(a) Water temperature

20±1℃

(b) Dissolved oxygen concentration

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

(c) pH

The test was performed without adjusting pH.

(d) Irradiation condition

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

(e) Feeding

Test organisms were not fed during the exposure.

3.5 Preparation of test solution

No correction with purity was done for the preparation of the test concentration. The test sample was employed in terms of volume using the density [1.678 g/cm^3(20°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 approximately 48 hours under 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for approximately 1 hour and then stock solution was prepared by taking out from the middle layer of the settled solution, and mixed and stirred with dilution water[*3] in a container for preparation to prepare test solution. The test solution was immediately divided into each test vessel and covered with glass lid not to produce head space. The added amount of the stock solution for each exposure level is shown below.

*3 similar water used in the control.

Exposure level Content of stock solution (%)	Added amount of stock solution (mL/1300mL)
Control	-
9.53	123.9
17.1	222
30.9	402
55.6	723
100	Stock solution was used for test solution as it was, and divided into each test vessel.

3.6 Observation and measurements

(1) Observation of test organisms

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

(2) Appearance of test solution

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

(3) Condition of test solutions

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

(4) Concentration of test item in test solution

The concentration of the test item in the test solutions was measured at the start of the exposure, before and after the renewal, and the end of the exposure (twice of a set of preparation and 24 hours after). At the start of the exposure, another solution sampled from the container for preparation

was used for analysis. At 24 hours after the preparation, the test solution for analysis was taken out with equal volume from the middle layer of the test solution in test vessels in each test level, and mixed. The concentration of the test item was analyzed by gas chromatography (GC). Analytical method and measured concentration of test item is shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(5) Solubility of test item in dilution water

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

3.7 Calculating method of EC_{50} *4

The EC_{50} value was estimated by Probit analysis for 24 hours and by Moving average analysis for 48 hours, and their 95% confidence intervals were estimated. The slope of the dose-response curve was also calculated for 24 hours.

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

3.8 Validity of the test

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

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

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

3.9 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401 rule B, 1999.
(JIS ; Japanese Industrial Standards)

4. Results and discussion

The comparison of content of stock solution to geometric mean of measured concentration is shown below.

Exposure level Content of stock solution (%)	Geometric mean of measured concentration (mg/L)
9.53	1.33
17.1	2.44
30.9	4.24
55.6	7.72
100	14.1

The following test concentrations are expressed using a geometric mean of the measured concentrations.

4.1 Immobility

Minimum concentration causing 100 % immobility and maximum concentration causing no immobility at 48 hours were 14.1 mg/L and 1.33 mg/L, respectively. Immobility at 24 and 48 hours are shown in Table 1, and concentration-immobility curve is shown in Figure 1. In the control, no trapping daphnids at the water surface was observed, and immobility during the exposure was 0%, which meets the criterion for the validity of the test (i.e. not more than 10%).

4.2 Observed abnormal response

There was no abnormal response in the control.

The following results of observation were based on the comparison with the control organisms. The abnormal responses obtained in the test level during exposure were lethargic, immobility, hyper activity, and reduced activity. The result of the observation during exposure is shown in Table 2.

4.3 Observation and measurement of test solution

(1) Appearance of test solution

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

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature of the test solution during the exposure were 8.3 to 8.4 mg/L, 7.4 to 7.5, and 19.5 to 20.0℃, respectively. Conditions of the test solutions are shown in Table 3-1, 3-2 and 3-3. The measured values of dissolved oxygen concentration met the criterion for the validity of the test (more than 3 mg/L at the end of exposure).

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 1.30 to 15.5 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 1.31 to 13.5 mg/L which were 77.3 to 102% of the concentration at the preparation. The result of the measured concentrations of the test item is shown in Appendix 2.

4.4 EC_{50}

The 24-hour and 48-hour EC_{50}s of 13F-EtOH to *Daphnia magna* were 10.4 mg/L (95% confidence interval: 9.01 to 12.2 mg/L) and 8.20 mg/L (95% confidence interval: 7.16 to 9.46 mg/L), respectively. The EC_{50}s at each observation time are shown in Table 4.

4.5 Discussion

This study was conducted to estimate EC_{50} for the test organisms under the solubility in the dilution water. The measured concentrations of the test item in the test solution were maintained in the range of 77.3 to 102% of the prepared concentration under semi-static regime (renewal at 24 hours after). Since the environmental conditions were within the suitable range, 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 Immobility

Measured Concentration*5 (mg/L)		Immobility (%)			
		24 hour		48 hour	
		Replicate	Test level	Replicate	Test level
Control	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	
1.33	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	
2.44	A	0	0	0	5
	B	0		0	
	C	0		0	
	D	0		20	
4.24	A	0	0	0	5
	B	0		0	
	C	0		0	
	D	0		20	
7.72	A	0	15	20	30
	B	20		20	
	C	0		40	
	D	40		40	
14.1	A	100	85	100	100
	B	40		100	
	C	100		100	
	D	100		100	

*5: geometric mean of measured concentration

(The followings are expressed as measured concentration)

Table 2 Observed abnormal response

Measured concentration (mg/L)	Observed abnormal response	
	24 hours	48 hours
Control	-	-
1.33	-	HYP
2.44	-	HYP IM LETH
4.24	-	IM HYP
7.72	IM LETH RA	HYP IM LETH RA
14.1	IM LETH RA	IM LETH

- : No abnormal response

Abbreviation of symptom

HYP: Hyper active

IM : Immobilization

LETH: Lethargic

RA : Reduced activity

Table 3-1 Dissolved oxygen concentration of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	8.3	8.4	8.3	8.3
1.33	8.4	8.4	8.4	8.3
2.44	8.4	8.4	8.4	8.3
4.24	8.3	8.4	8.4	8.3
7.72	8.3	8.4	8.4	8.3
14.1	8.3	8.4	8.4	8.3

Unit : mg/L

Table 3-2 pH of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	7.4	7.4	7.5	7.5
1.33	7.5	7.4	7.5	7.5
2.44	7.5	7.5	7.5	7.5
4.24	7.5	7.5	7.5	7.5
7.72	7.5	7.5	7.5	7.5
14.1	7.5	7.5	7.5	7.5

Table 3-3 Temperature of test solutions

Measured concentration (mg/L)	At the start	24 hours		At the end
		Before the renewal	After the renewal	
Control	20.0	19.9	19.9	19.5
1.33	20.0	19.9	19.9	19.5
2.44	20.0	19.9	19.9	19.5
4.24	20.0	19.9	19.9	19.5
7.72	20.0	19.9	19.9	19.5
14.1	20.0	19.9	19.9	19.5

Unit : °C

Table 4 EC_{50} to *Daphnia magna*

Exposure duration	EC_{50} (mg/L)	95% confidence interval (mg/L) (Slope of the dose-response curve)	Statistical procedure used for determination of EC_{50}
24-hour	10.4	9.01 to 12.2 (8.00)	Probit analysis-
48-hour	8.20	7.16 to 9.46 (-)	Moving average

- : Not obtained

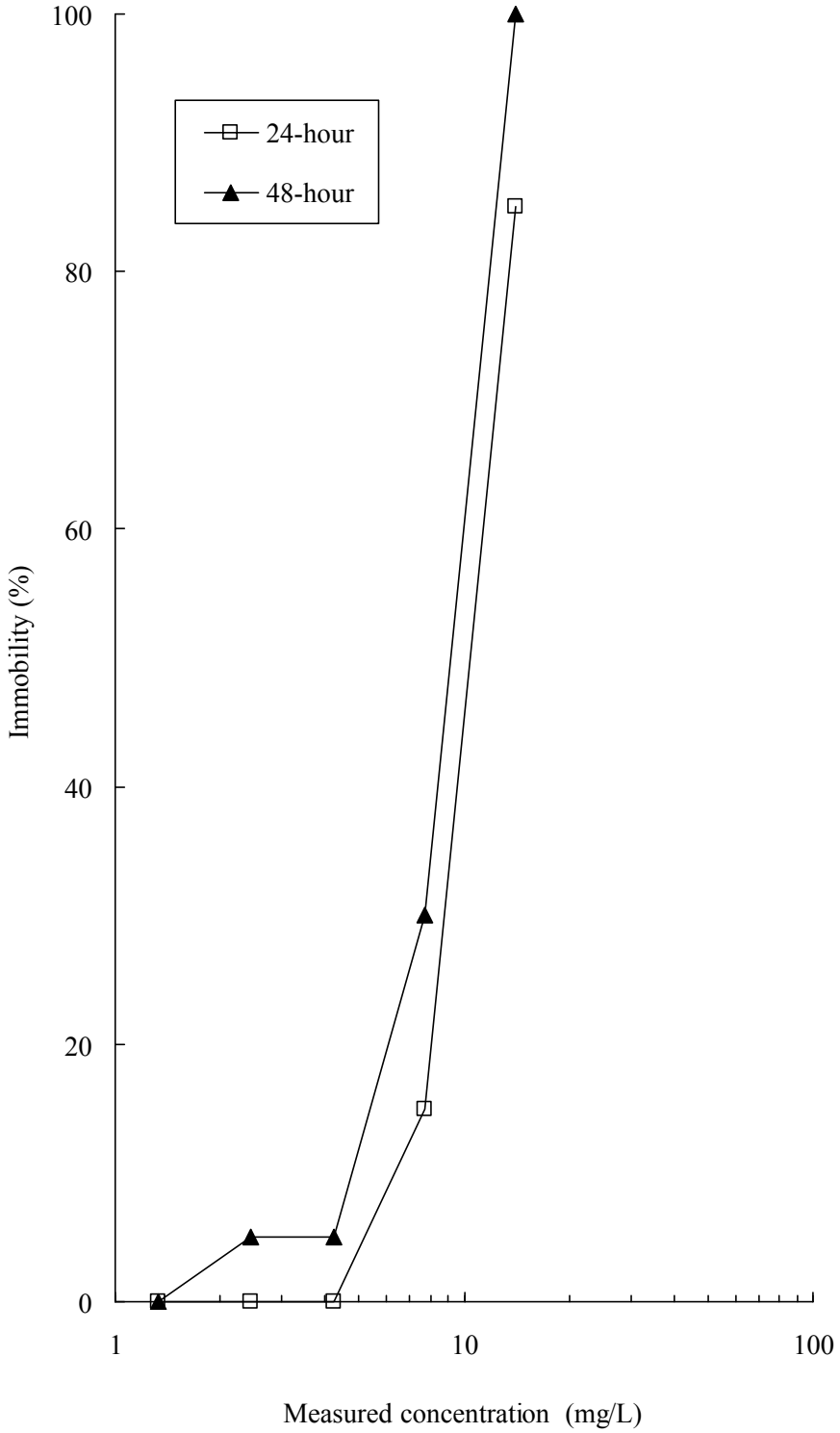


Figure 1 Concentration-immobility curve.

Appendix 1

Chemical characteristics of dilution water

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

Parameter	Unit	Results	Lower limit of
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

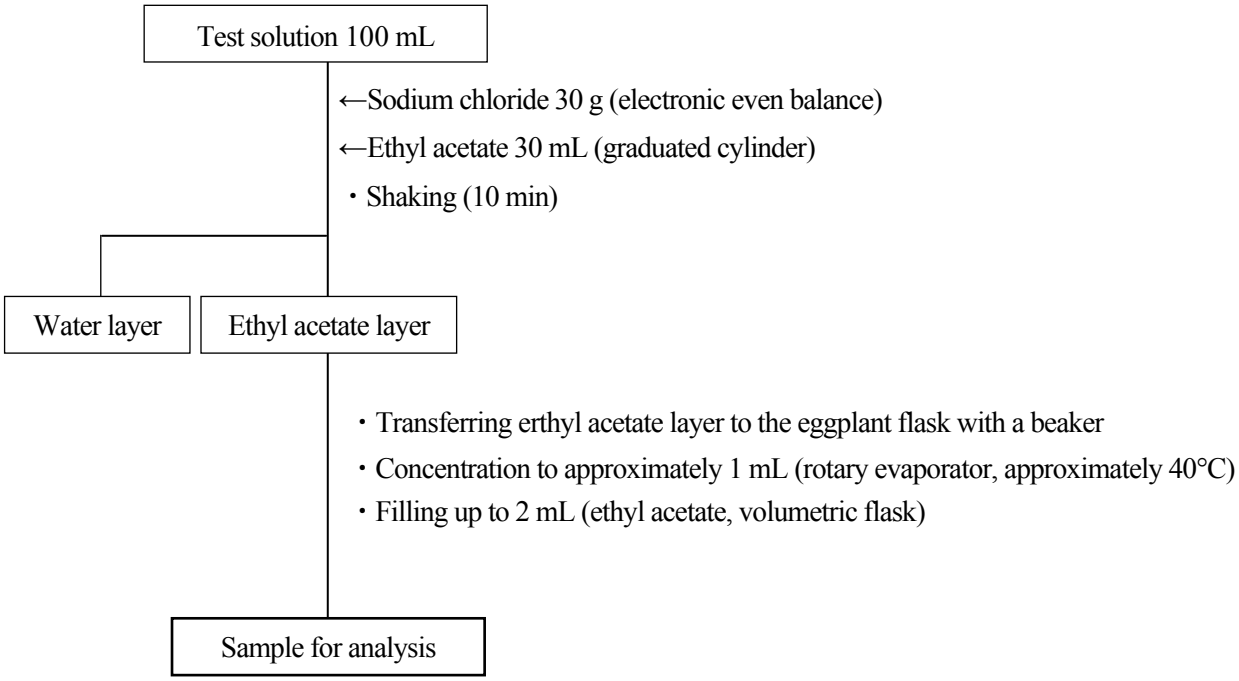
①30.9, 55.6 and 100%

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

②Control, 9.53 and 17.1%

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.

①Analytical conditions (30.9, 55.6 and 100%)

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(1 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

②Analytical conditions (Control, 9.53 and 17.1%)

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.

①30.9, 55.6 and 100%

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.

②Control, 9.53 and 17.1%

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

①30.9, 55.6 and 100%

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.

②Control, 9.53 and 17.1%

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.

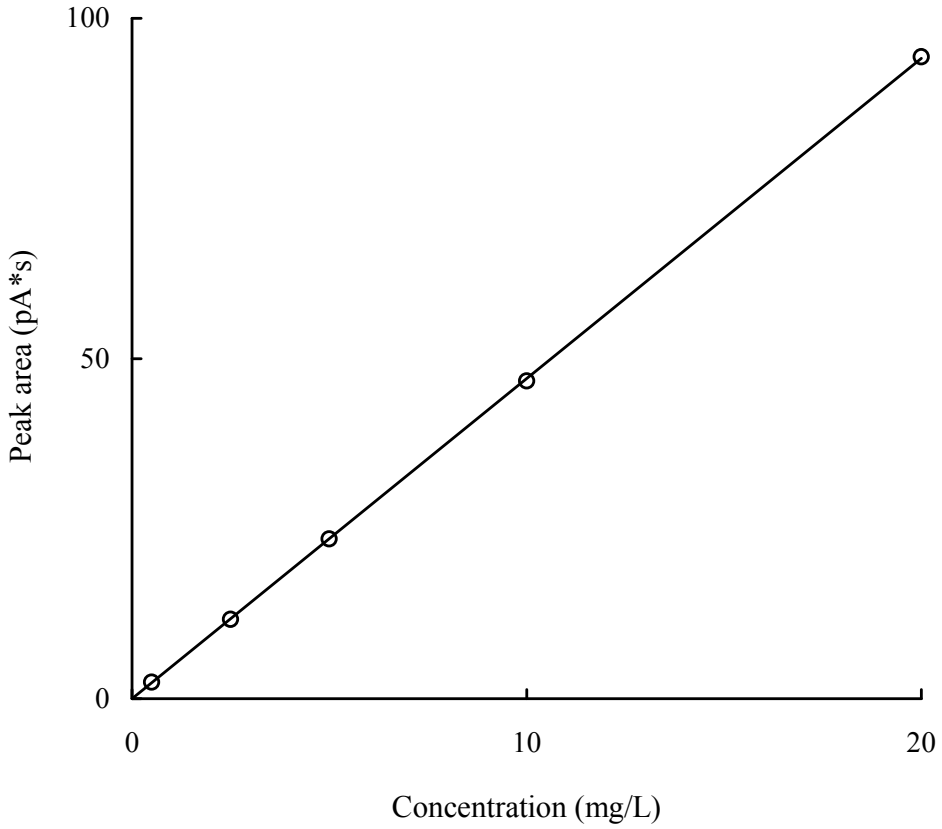
Appendix table 2-1 Measured concentrations of test item in test solutions

Stock solution (%)	Measured concentration (mg/L) (Percentage of measured concentration versus that at preparation %)				
	At the start	24 hours		At the end	Geometric mean
		Before the renewal	After the renewal		
Control	n.d.	n.d.	n.d.	n.d.	
9.53	1.30	1.33 (102)	1.37	1.31 (95.1)	1.33
17.1	2.62	2.36 (90.1)	2.54	2.25 (88.7)	2.44
30.9	4.39	4.28 (97.4)	4.37	3.94 (90.1)	4.24
55.6	8.46	6.98 (82.5)	8.82	6.82 (77.3)	7.72
100	14.4	13.3 (92.7)	15.5	13.5 (87.0)	14.1

n.d. : < 0.0124 mg/L

Appendix 3

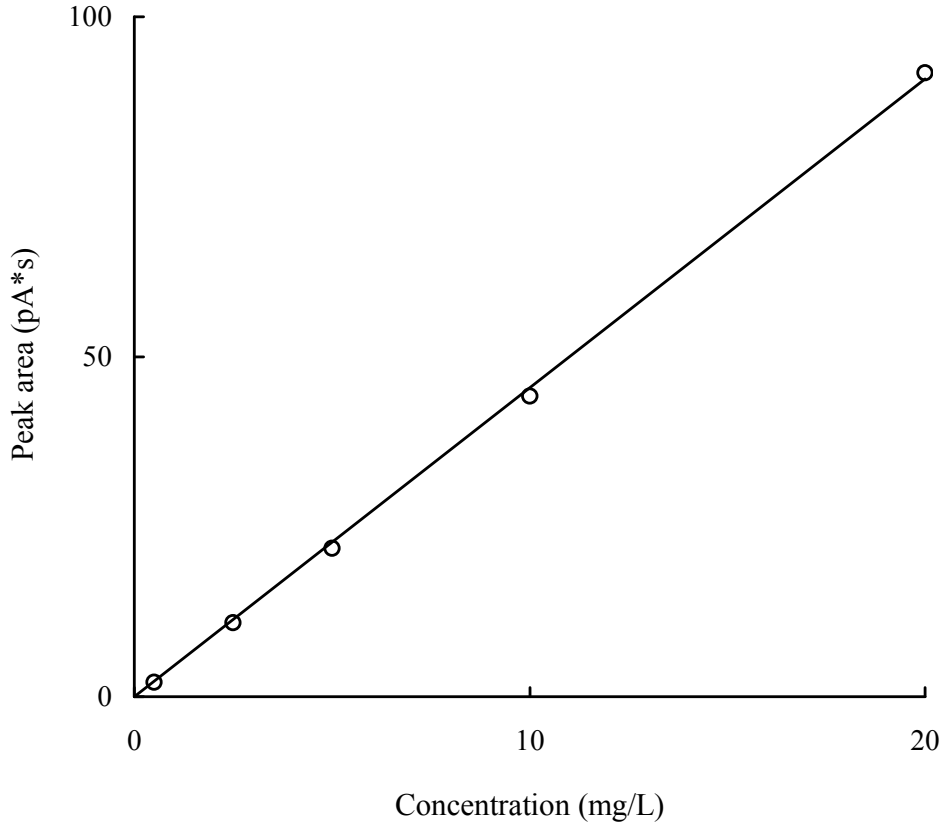
Calibration curve and chromatogram



y = 4.71x
r = 1.00

Concentration (mg/L)	Peak area (pA*s)
0.500	2.417
2.50	11.678
5.00	23.484
10.0	46.699
20.0	94.364

Appendix figure 3-1-1 Calibration curve of 13F-EtOH for analysis by GC. (Control, 9.53 and 17.1%).



y = 4.54x
r = 1.00

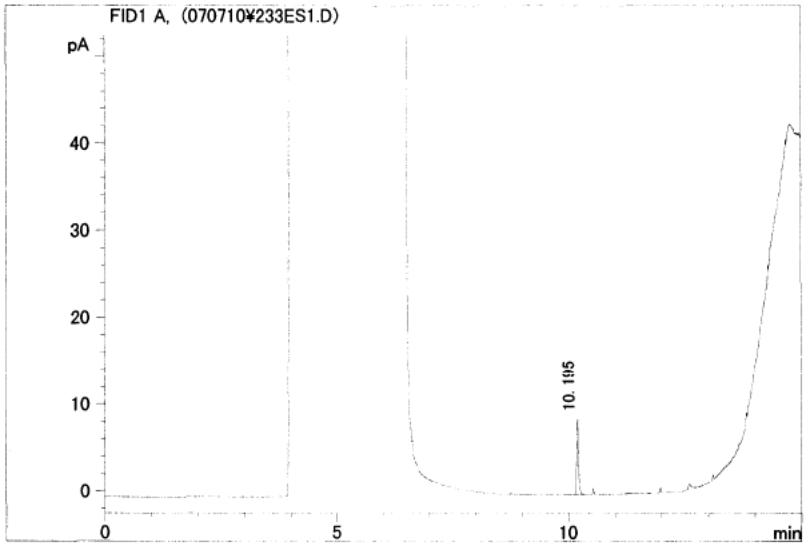
Concentration (mg/L)	Peak area (pA*s)
0.500	2.121
2.50	10.872
5.00	21.829
10.0	44.218
20.0	91.762

Appendix figure 3-1-2 Calibration curve of 13F-EtOH for analysis by GC(30.9, 55.6 and 100%).

Standard solution 5.00 mg/L

Study No. 94233

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¥070710¥233ES1.D
サンプル名 : std 5.00mg/L



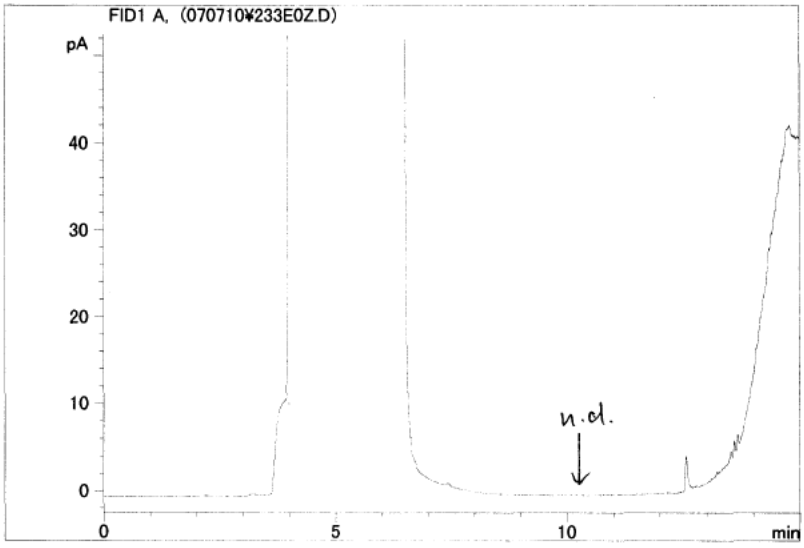
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	24.092	8.645	10.195

Appendix figure 3-2-1 GC chromatogram of standard solution at start of exposure (Control, 9.53 and 17.1%).

Control

Study No. 94233

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¥070710¥233E0Z.D
サンプル名 : Cont (*50) H0h



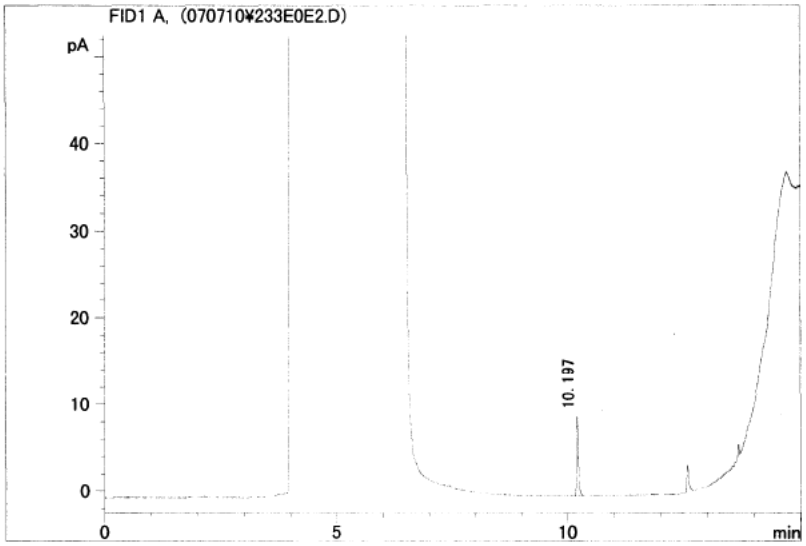
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

9.53% (Stock solution)

Study No. 94233

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¥070710¥233E0E2.D
サンプル名 : 9.53%(*50D10)H0h



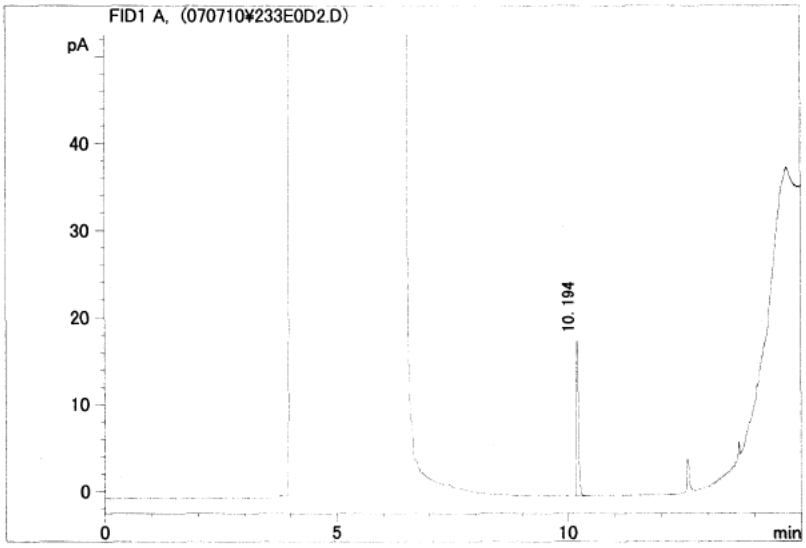
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	25.322	9.039	10.197

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

17.1% (Stock solution)

Study No. 94233

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¥070710¥233E0D2.D
サンプル名 : 17.1%(*50D10)H0h



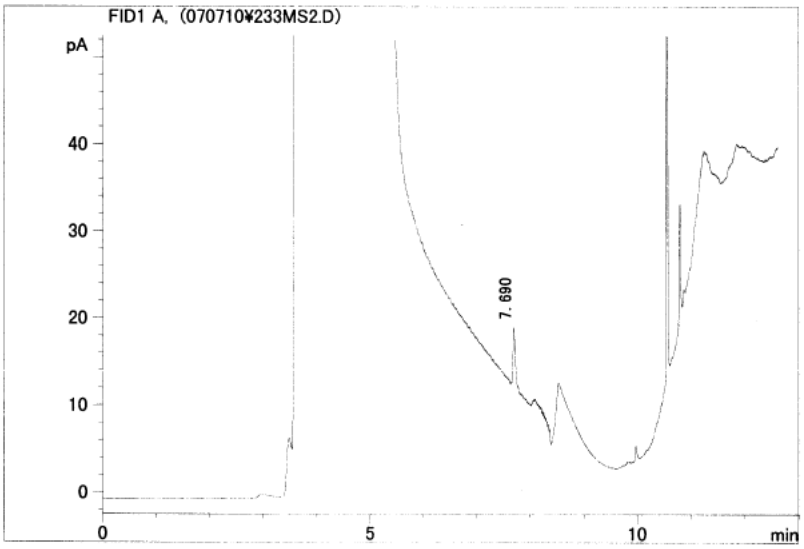
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	51.049	17.822	10.194

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

Standard solution 5.00 mg/L

Study No. 94233

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¥070710¥233MS2.D
サンプル名 : std 5.00mg/L



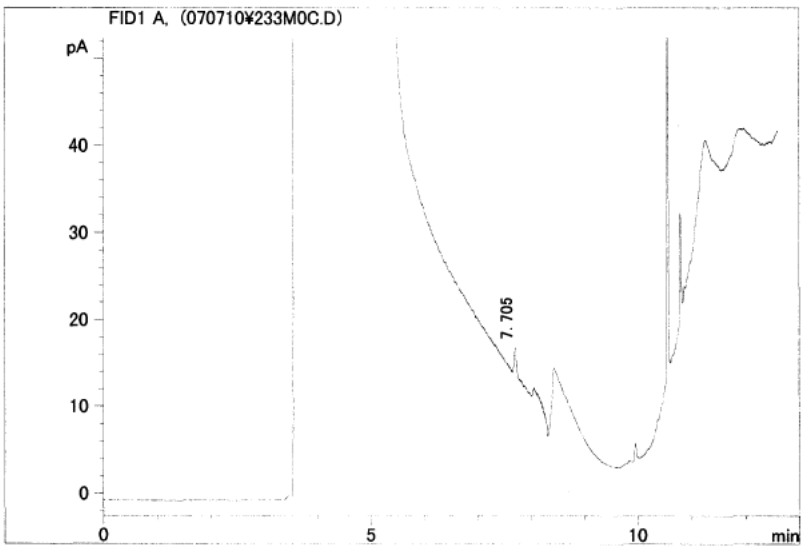
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	21.479	6.798	7.690

Appendix figure 3-2-5 GC chromatogram of standard solution at start of exposure (30.9, 55.6 and 100%).

30.9% (Stock solution)

Study No. 94233

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¥070710¥233M0C.D
サンプル名 : 30.9%(D2)H0h



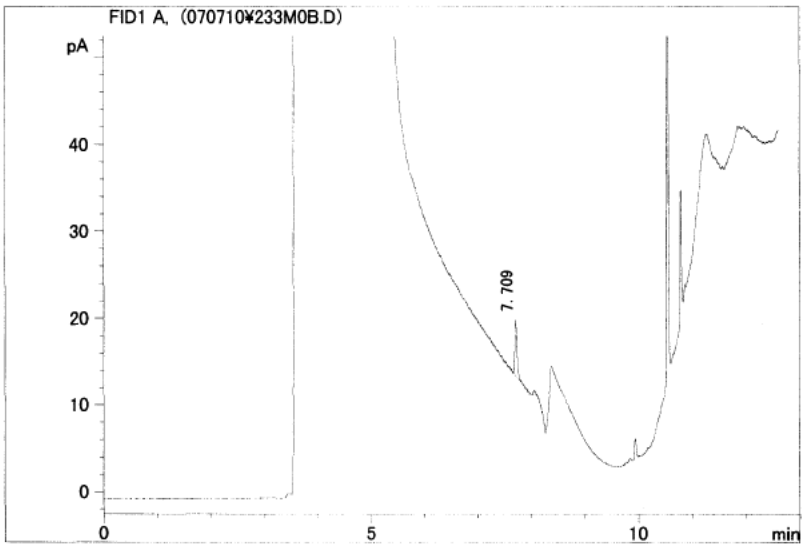
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	9.431	3.137	7.705

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

55.6% (Stock solution)

Study No. 94233

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¥070710¥233M0B.D
サンプル名 : 55.6%(D2)H0h



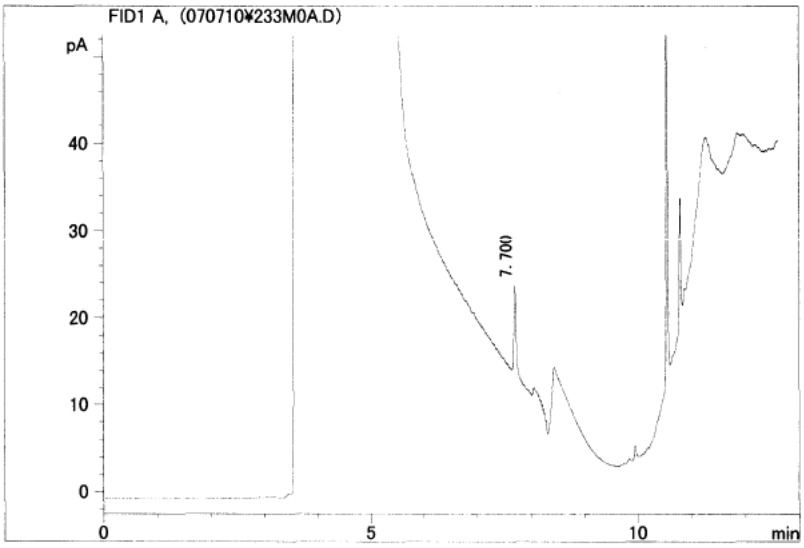
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	18.170	6.331	7.709

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

100% (Stock solution)

Study No. 94233

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¥070710¥233M0A.D
サンプル名 : 100%(D2)H0h



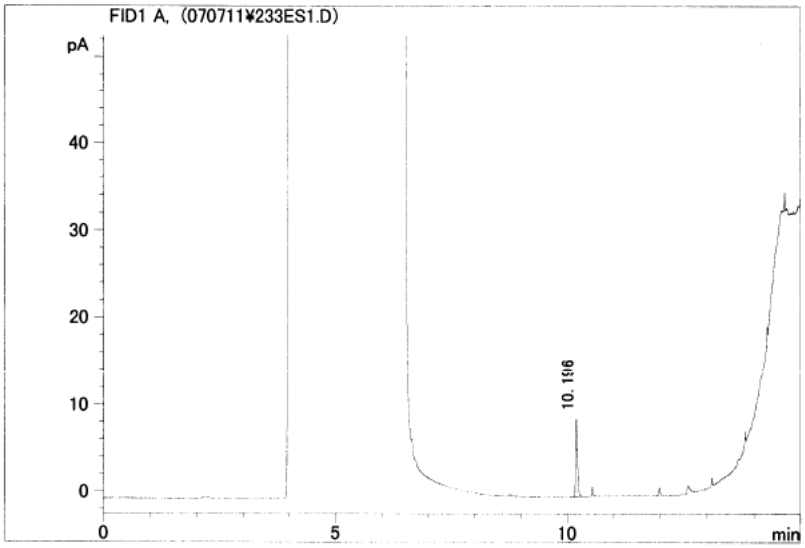
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	30.919	10.071	7.700

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

Standard solution 5.00 mg/L

Study No. 94233

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¥070711¥233ES1.D
サンプル名 : std 5.00mg/L



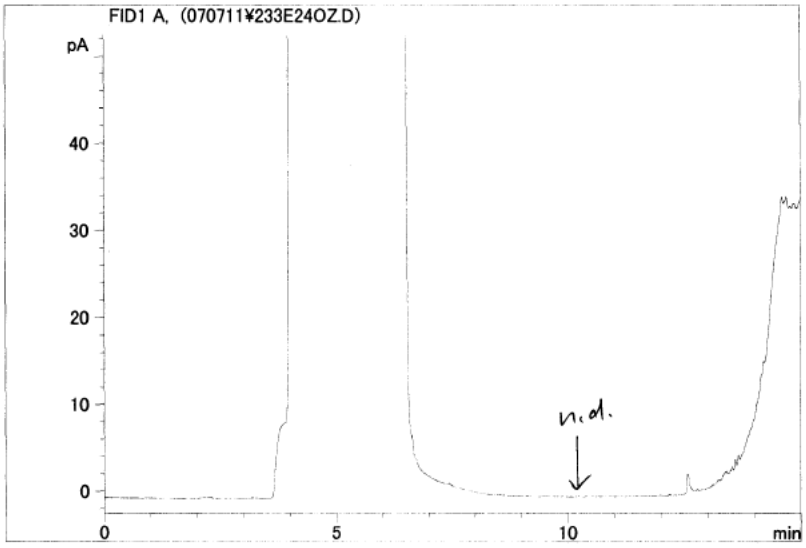
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	24.452	8.986	10.196

Appendix figure 3-3-1 GC chromatogram of standard solution before renewal at 24 hours (Control, 9.53 and 17.1%).

Control

Study No. 94233

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¥070711¥233E24OZ.D
サンプル名 : Cont ~~(*50)H24New~~
(*50)H24 Old
メカニカルミス
2007.7.12



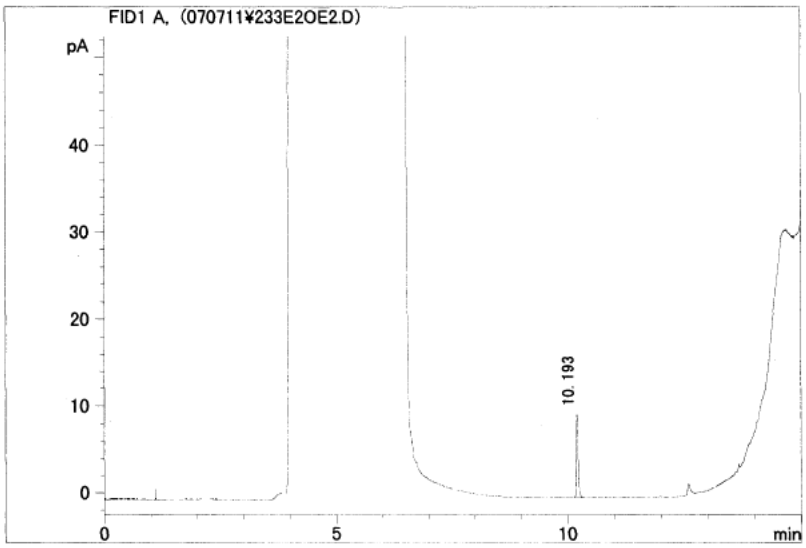
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

9.53% (Stock solution)

Study No. 94233

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¥070711¥233E20E2.D
サンプル名 : 9.53% ~~(¥80) H24New~~
(¥500) H24 0W
入れ込みより
2007.7.12



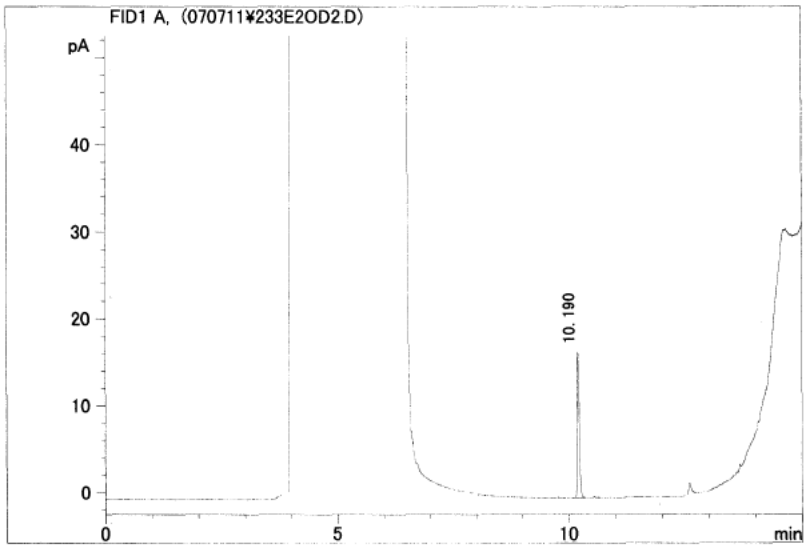
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	26.336	9.599	10.193

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

17.1% (Stock solution)

Study No. 94233

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¥070711¥233E20D2.D
サンプル名 : 17.1% ~~(*50)H24Now~~ (*50D10)H24 Old
入力ミスにより訂正
2007.7.12



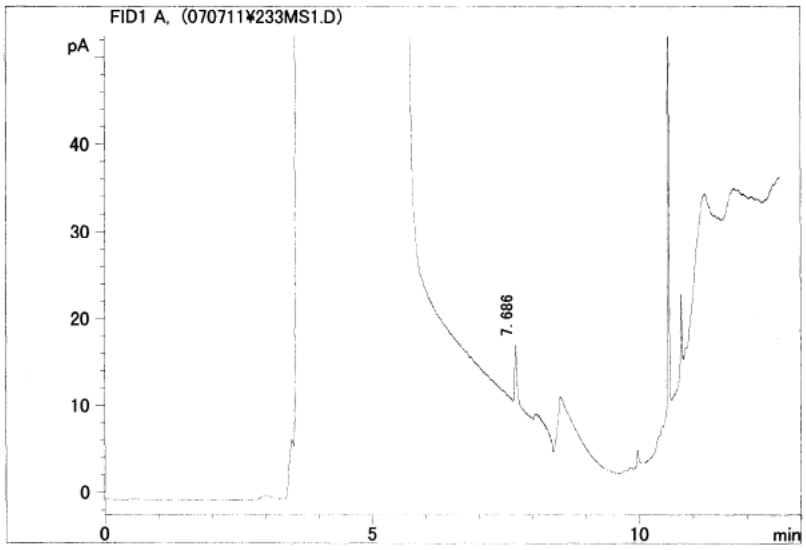
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	46.699	16.752	10.190

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

Standard solution 5.00 mg/L

Study No. 94233

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¥070711¥233MS1.D
サンプル名 : std 5.00mg/L



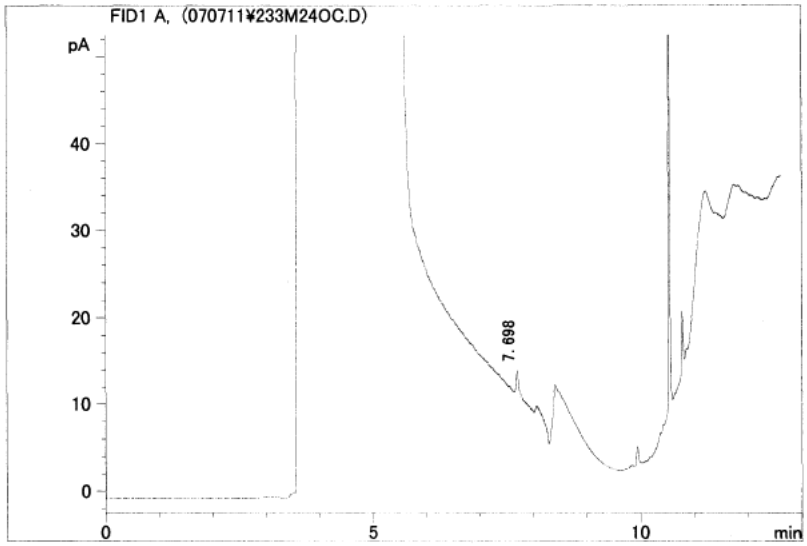
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	21.101	6.796	7.686

Appendix figure 3-3-5 GC chromatogram of standard solution before renewal at 24 hours (30.9, 55.6 and 100%).

30.9% (Stock solution)

Study No. 94233

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¥070711¥233M240C.D
サンプル名 : 30.9%(D2)H240Id



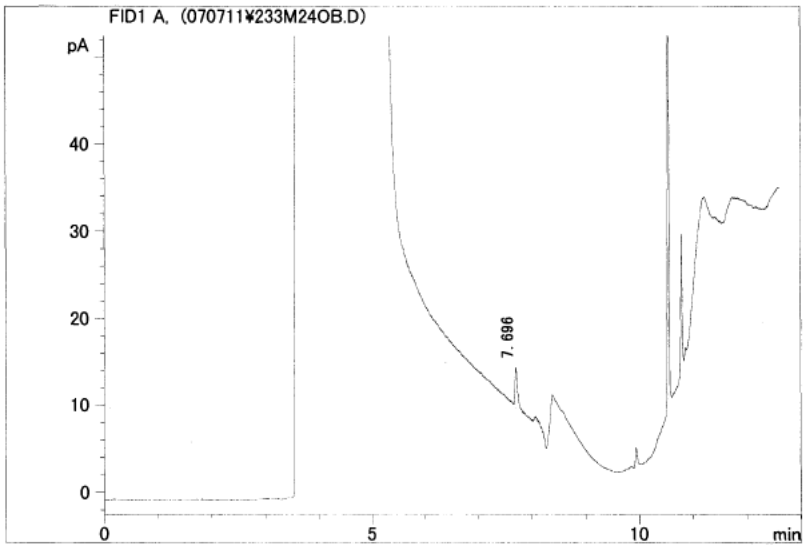
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	9.028	2.825	7.698

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

55.6% (Stock solution)

Study No. 94233

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¥070711¥233M240B.D
サンプル名 : 55.6%(D2)H240Id



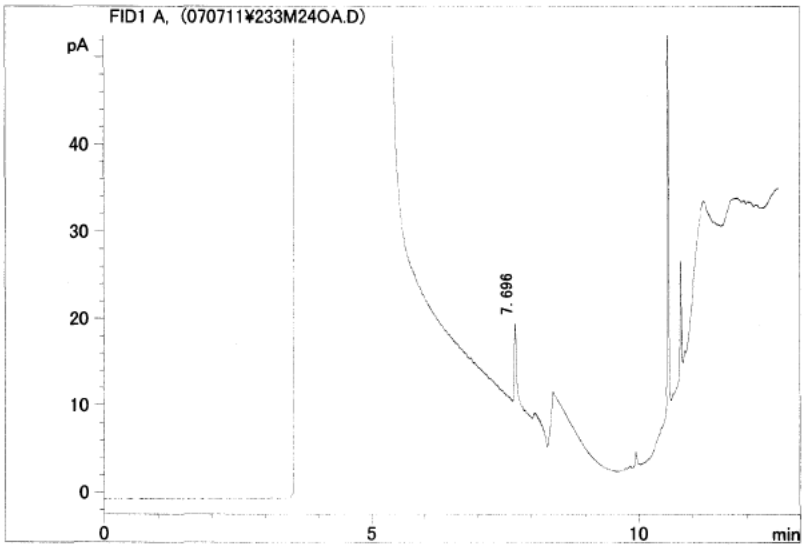
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	14.718	4.689	7.696

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

100% (Stock solution)

Study No. 94233

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¥070711¥233M240A.D
サンプル名 : 100%(D2)H240Id



#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	28.145	9.205	7.696

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 : approximately 600 mL)

4.2 Test conditions

(1)Test temperature : 20±1°C

(2)The number of measurement : Twice (after the mixture was stirred for 24 and 48hours)

(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 approximately 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 hours or 48 hours, the flask was settled in a water bath for approximately 1 hour.

(4) After settling, the samples were quantitatively analyzed to measure the concentration of the test item. In addition, three repetitions per sample were analyzed.

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 18.0 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	12.1	12.7	11.9
	b	12.7		
	c	13.5		
Sample-2	a	8.82	10.0	
	b	10.7		
	c	10.5		
Sample-3	a	12.7	12.9	
	b	13.1		
	c	12.8		

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	17.6	17.1	18.0
	b	16.9		
	c	16.8		
Sample-5	a	16.7	16.9	
	b	17.7		
	c	16.4		
Sample-6	a	18.2	19.9	
	b	21.1		
	c	20.4		

*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 (20±1°C) for 24 and 48 hours. And then the middle layer was sampled after settling for approximately 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	21.2	22.4
	②	21.2	
	③	24.8	
Sample-2	①	15.4	13.7
	②	13.1	
	③	12.7	

Value measured after stirring for 48 hours

Sample name		Measured value(mg/L)	Arithmetic mean value (mg/L)
Sample-3	①	16.9	17.1
	②	18.0	
	③	16.5	
Sample-4	①	19.2	19.8
	②	19.6	

	③	20.7	

Solubility of test item in dilution water was around 12 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 12 to 25 mg/L. The test solutions were gently stirred. And then, the middle layer was sampled after settling for approximately 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. Study for effect on test organism

1) Preliminary study 1

(1) Method

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for approximately 48 hours under 20±1°C to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for approximately 1 hour, and then stock solution was prepared by taking out from the middle layer of the settled solution. Test solution was prepared by appropriately diluting the stock solution with the dilution water. The preliminary study to investigate the effect of the test item on the test organisms was performed under closed system. 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.

(2)Result

Content of stock solution (mg/L)	24 hours		48 hours	
	Immobility (%)	Others	Immobility (%)	Others
10.0	0	-	0	HYP
30.0	0	-	0	HYP RA
60.0	60	HYP RA	80	HYP
100	100	-	100	-

Exposure: Semi-static regime (renewal at 24 hours after), closed system
The number of organisms: Ten daphnids/test level (five daphnids/test vessel),
- shows that no other abnormal response was observed.
Abbreviation of symptom
HYP: Hyper active
RA : Reduced activity

The exposure level of 100% content of the stock solution resulted in 100% immobility. Although no immobility was observed in 10% content level, some individuals showed abnormal response of hyper active behavior.

2) Preliminary study 2

(1) Method

The effect of the test item on the test organisms was investigated again under static and semi-static (renewal at 24 hours after) and closed system with the test solution prepared by the same procedure as used in the preliminary study 1. The concentration of the test item in the test solution was also carried out.

(2) Result

Content of stock solution (mg/L)		24 hours		48 hours	
		Immobility (%)	Others	Immobility (%)	Others
Static	10.0	0	-	0	-
	100	100	-	100	-
Semi-static	10.0	0	-	0	-
	100	100	-	100	-

The number of organisms:

10.0% content; 10 daphnids/test level (five daphnids/test vessel)

100% content; 5 daphnids/test level (five daphnids/test vessel)

- shows that no other abnormal response was observed.

The exposure level of 100% content of the stock solution resulted in 100% immobility. No immobility and no abnormal response were observed in 10% content level.

Content of stock solution (mg/L)	Measured concentration (mg/L) (percentage of measured concentration at start)		
	At the start	after 24 hours	At the end (after 48 hours)
10.0	1.44	1.58 (110)	1.59 (110)
100	18.1	16.5 (91.4)	10.2 (56.6)

While the measured concentration of the test item in the test solution at 10.0% content of the stock solution was almost stable, that at 100% content of the stock solution tended to decrease.

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

In the exposure level of the 100% content of the stock solution, 100 % of immobility was observed, and no immobility was observed in the level of 10.0% content. The preliminary study was carried out under closed system since the test item was anticipated volatility, however, the concentration of the test item in the test solution of 100% content level of the stock solution tended to decrease during the exposure. Therefore, the definitive study was planed to be conducted with semi-static replacement regime.

3. Operation of definitive study

1) Measurement of solubility of test item in dilution water

Based on the result of the preliminary study, the measurement of the solubility was carried out using the solution prepared by mixing the test sample and the dilution water to produce approximately 100 mg/L, and by stirring gently for 24 and 48 hours under closed system and 20±1℃. 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 approximately 1 hour after cease of stirring. The concentration of the test item was measured for the prepared test solution.

2) Definitive study

From the result of the preliminary studies, the definitive study was planed to be carried out using 5 exposure levels of 100, 55.6, 30.9, 17.1 and 9.53% content (a geometric series with a factor of 1.8) of stock solution and a control under semi-static regime (renewal at 24 hours after). The stock solution, which was removed insoluble substance, was prepared with the same procedure, of approximately 48-hour stirring, of test solution as used in the preliminary study of measurement of solubility. The test solution was prepared by appropriately diluting the stock solution with the dilution water. No correction of purity was employed for the preparation of concentration. The measurement of the test item 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.