Receipt number	662-06-E-4232
Study number	94232

FINAL REPORT

Algae Growth Inhibition Study of 13F-EtOH with *Pseudokirchneriella subcapitata*

July 25, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Algae Growth Inhibition Study of 13F-EtOH with *Pseudokirchneriella subcapitata*

Study number 94232

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94232, issued on July 25, 2007).

The Study Director was changed from to because had been reshuffled.

Date

Study Director _____

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Algae Growth Inhibition Study of 13F-EtOH with *Pseudokirchneriella subcapitata*

Study number 94232

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

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
(2) "OECD Principles of Good Laboratory Practice (November 26, 1997, ENV/MC/CHEM (98)17)"

This final report reflects the raw data accurately and it has been confirmed that the test data are valid.

Date July 25, 2007

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

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Study number 94232

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 12, 2007	July 12, 2007
Measurement of solubility	July 11, 2007	July 18, 2007
	July 13, 2007	July 18, 2007
Start of the exposure and after the exposure	July 13, 2007	July 18, 2007
	July 15, 2007	July 18, 2007
	July 18, 2007	July 18, 2007
Raw data and final report draft	July 24, 2007	July 25, 2007
Final report	July 25, 2007	July 25, 2007

Date July 25, 2007

Quality Assurance Unit, Head Signed in original

CONTENTS

Title Algae Growth Inhibition Study of 13F-EtOH with *Pseudokirchneriella subcapitata*

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 determine the effect of 13F-EtOH on growth of algae.

Test method The study was performed according to the following test methods and guidance document.

(1) Algal Growth Inhibition 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, Partial amendment November 20, 2006)

(2) OECD Guidelines for Testing of Chemicals, Section 2: Effects on Biotic Systems, “Freshwater Alga and Cyanobacteria, Growth Inhibition Test (Guideline 201, 23 March 2006)”

(3) OECD Guidance Document 23 “Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures” (September 2000)

Applied GLP The 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 11, 2007
Solubility study starting date	July 11, 2007
Bioassay starting date	July 15, 2007
Experimental completion date	July 18, 2007
Solubility study completion date	July 13, 2007
Bioassay completion date	July 18, 2007
Study completion date	July 25, 2007

Storage of test item, raw data, etc.

(1) Test item

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

Personnel

Study Director :
Section 4 (Eco-toxicity test area)

Study personal
Biology :

Analysis :

Approval of final report
Study Director Date July 25, 2007

Signature Signed in original

SUMMARY

Title

Algae Growth Inhibition Study of 13F-EtOH with *Pseudokirchneriella subcapitata*

Test condition

(1) Test item	13F-EtOH
(2) Test organism	*Pseudokirchneriella subcapitata*
(3) Exposure duration	72 hours
(4) Test concentration	Five exposure levels of 100, 31.6, 10.0, 3.16 and 1.00% of stock solution content (a geometric series with a factor of $\sqrt{10}$) and control
(5) Type of test	Incubation with shaking (approximately 100 rpm)
(6) Preparation of test solution	The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration), and they were stirred under closed system with little headspace for approximately 48 hours. After settlement for 1 hour, stock solution was prepared by taking out from the middle layer. Then the test solution was prepared by diluting the stock solution with medium accordingly.
(7) Replicate	Six replicates / control level Three replicates / exposure level
(8) Volume of test solution	600 mL / control level (100 mL / test vessel) 300 mL / exposure level (100 mL / test vessel)
(9) Temperature in incubator	21 to 24°C, not varied more than ± 2°C
(10) Light condition	Continuous illumination using a fluorescent light [The measured light intensity was 60 to 120 μE/m^2/s (not varied more than 20%) at the level of the test solutions during exposure period.]
(11) Measurement of cell growth	Cell concentration
(12) Analysis of concentration of test item in test solution	GC analysis (at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure)

Results

(1) Solubility of test item in medium (23 ± 1°C)	17.4 mg/L
(2) Concentration of test item in test solution (versus at the start of exposure)	
At the start of the exposure	0.0966 to 9.45 mg/L
24 hours after the start of the exposure	0.0436 to 5.02 mg/L (43.1 to 58.6%)
48 hours after the start of the exposure	0.0466 to 4.69 mg/L (41.2 to 60.2%)
At the end of the exposure	0.0445 to 3.73 mg/L (39.4 to 51.1%)
(3) EC_{50} (E_rC_{50})	> 5.19 mg/L
(4) NOEC (Growth rate 0-3d)	1.47 mg/L

[The above-mentioned concentrations (3) and (4) are based on geometric mean of measured concentrations.]

Conclusion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at around or lower the solubility of the test item in medium. As a result, the EC_{50} (E_rC_{50}) was >5.19 mg/L and the NOEC was 1.47 mg/L. The concentration of the test item in the test solution of the highest exposure level at the start of exposure was low (9.45 mg/L) compared with the solubility in the medium (17.4 mg/L). However, considering the variation among test vessels for the measurement of the solubility (14.0 to 21.4 mg/L), it was judged that the concentration of test item at the preparation was around solubility in medium. The concentrations of test item in the test solutions were significantly decreased during the exposure. The reason was estimated to the limit in algae growth inhibition study of the volatile substance (volatilization to headspace in the test vessel).

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

Pseudokirchneriella subcapitata (ATCC 22662)
(The old scientific name "*Selenastrum capricornutum*")

(2) Reason for selection of species

Species recommended in the test guideline

(3) Source

Pseudokirchneriella subcapitata which originally came from the American Type Culture Collection (12301 Parklawn Drive Rockville, Maryland 20852-1776 U.S.A.) on Jun. 30, 1995 and has been cultured in this laboratory was used. An algae growth inhibition test of potassium dichromate (Reagent grade, Wako Pure Chemical Industries, Ltd.) with the test organisms was conducted to confirm the reproducibility of the test system (on May 21 - May 24, 2007). The E_rC_{50} (0-3d) of potassium dichromate was 0.900 mg/L. This value was within the normal range of the reference substance in this laboratory (mean ± 2S.D.: 0.698 to 1.08 mg/L) [mean ± S.D.: 0.891 ± 0.097 mg/L (n=3)].

3.2 Culture medium

The medium recommended in OECD test guideline (Guideline 201, 23 March 2006). The composition of medium is shown in Appendix 1. Medium was used under sterile condition.

3.3 Test apparatus and equipment

(1) Test apparatus

Test vessel : Sterilized 500 mL Erlenmeyer flask (closed vessel)

(2) Test equipment

Incubator : Incubator with temperature control, continuous shaking and continuous illumination, maintained the uniform light intensity (Incubator with rotary shaker and artificial illumination, USI CO., LTD)

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 added to medium. The test vessels that contain test solution and test organisms were shaken (approximately 100 rpm) during the exposure.

(b) Exposure duration

72 hours

(c) Test concentration

Five exposure levels of 100, 31.6, 10.0, 3.16 and 1.00% of the stock solution content (a geometric series with a factor of $\sqrt{10}$) prepared by the method described in section 3.5 were used in the test. The test concentrations and the factor were decided based on the results of preliminary studies. The results of preliminary studies are shown in Additional data.

(d) Control

The medium without the test item, which was treated in the same manner as the test solution (except the collection of the solution from the middle layer) was used as the control.

(e) Replicates

Six replicates / control level

Three replicates / exposure level

(f) Initial cell concentration

The pre-culture, incubated under the same conditions as the test for 3 days and exponentially growing was used as inoculum to prepare the initial cell concentration of approximately 5×10^3 cells/mL.

(g) Operation

All operations were carried out under sterile conditions.

(h) Volume of test solution

600 mL / control level (100 mL / test vessel)

300 mL / exposure level (100 mL / test vessel)

(2) Conditions of test environment

(a) Temperature in the incubator

21 to 24°C, not varied more than ± 2°C

(b) Light

Continuous illumination provided with 60 to 120 μE/m^2/s (fluctuation range: mean ± 20%) at the level of the test solutions, using a fluorescent light with wavelength range of 400 to 700 nm.

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 and medium were mixed to prepare approximately 100 mg/L (nominal concentration). Then, they were stirred by magnetic stirrer for approximately 48 hours in the closed bottle with little headspace. After the mixture was left at rest for approximately 1 hour, the saturated solution of test item was collected from the middle layer for use as the test solution. Then, required volume of the stock solution and medium[*3] were added to the preparing vessel and mixed to prepare the test solution. The test solution was divided into each test vessel.

The added amount of the stock solution for the preparation in each exposure level is shown

Exposure level Content of stock solution (%)	Added amount of stock solution (mL / 800 mL)
Control	-
1.00	8.00
3.16	25.3
10.0	80.0
31.6	253
100	800

*3 The medium treated in the same manner as control

3.6 Observation and measurements

(1) Cell growth, etc.

Biomass was shown as cell concentration.

Cell concentration was counted with particle counter (Model COULTER Z1, Beckman Coulter) at 24, 48, and 72 hours after the start of the exposure. The blank value correction was conducted by measuring simultaneously the blank value of the blank solution (without algae) which was separately prepared when the test solutions were prepared. Furthermore, the cell condition for one vessel in each test level was observed under microscope (Model BX41, Olympus Co., Ltd.) at the end of the exposure.

(2) Appearance of test solution

The appearance of the test solutions was observed at the start and the end of the exposure.

(3) Water quality and environmental conditions

The pH of the test solution was measured at the start and end of the exposure. For the measurement of pH, another solution sampled from the vessel for preparation was used at the start of the exposure and one test vessel in each test level was used for the measurement at the end of exposure. The culture temperature and light intensity in the incubator were measured once a day during the exposure. The pH measurements were carried out on a portable pH meter (Model HM-21P, DKK-TOA Co.). The temperature was measured on a calibrated thermometer of glass stick type. Light intensity was measured on quantum scalar laboratory irradiance meter (Model LI-250A, LI-COR).

(4) Concentration of test item in the test solution

It was considered that the measurement was necessary with time from the results of the preliminary studies. Therefore, the concentration of test item in the test solution was measured at the start of the exposure, 24 and 48 hours after the start of the exposure, and the end of the exposure. At the start, the solution sampled from the container for preparation in each test level was used for the measurement. At 24 and 48 hours after the start of the exposure, required amount of the test solution was sampled from the test vessel for analytical chemistry. At the end of the exposure, equal volume of the test solution was taken out from the 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 are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(5) Solubility of test item in medium

Solubility of the test item in medium was measured in this study because the solubility was estimated to be below 100 mg/L. Detail of the measurement and results are shown in Appendix 4.

3.7 Treatment of results

The results of the study were estimated by geometric mean of measured concentration.

(1) Calculation of concentration-inhibition rates

The mean value of biomass for each test level was plotted against time to produce growth curves. Using this curve, inhibition rates were calculated comparing with control values on growth rate.

Comparison of growth rates

The specific growth rate for a specific period was calculated as the logarithmic increase in biomass according to the following formula:

$$\mu_{i\text{-}j} = \frac{lnN_j - lnN_i}{t_j - t_i}$$

where

$\mu_{i\text{-}j}$: specific growth rate from time i to j (normally day^{-1})
N_i : measured number of cells/mL at t_i, nominal number at start t_0
N_j : measured number of cells/mL at t_j
t_i : time of i^{th} measurement after beginning of test (day)
t_j : time of j^{th} measurement after beginning of test (day)

Specific growth rate over the exposure duration (0-72 h) was calculated for determination of EC_{50}. In control, specific growth rates for section-by-section were calculated for check of validity of the test.

The percentage inhibition of the cell growth at each exposure level (I_μ) was calculated as the difference between the average specific growth rate at control level (μ_c) and that at exposure level (μ_t) as:

$$I_\mu = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

(2) Estimation of EC_{50} *4

The EC_{50} was estimated as "> the highest concentration of the test item" since no less than 50% of inhibition rate was not obtained within the exposure levels. The EC_{50} was denoted as E_rC_{50} based on growth rate.

*4 EC_{50} (Median Effective Concentration) is the concentration of the test item that results in 50% reduction in growth of the test organisms during the exposure.

(3) Estimation of No Observed Effect Concentration (NOEC[*5])

Regarding the growth rate, Bartlett's test was done to determine the homogeneity of variance for the data. Then one-way analysis of variance and Dunnett's multiple comparison test were used to determine the significant difference between the control level and exposure levels. NOEC was determined by the results of statistical analysis and cell condition.

*5 NOEC (No Observed Effect Concentration) is the concentration of the test item that does not cause any observed adverse effects on growth of the test organisms during the exposure.

3.8 Validity of the test

(1) The cell growth in the control cultures should have increased by a factor of at least 16 within the 72-hour exposure period.

(2) The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.

(3) The coefficient of variation of average specific growth rates in replicate control cultures must not exceed 7%.

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 contrast table of content percentages of the stock solutions at each exposure level and geometric mean of the measured concentrations are shown below.

Exposure level Content of stock solution (%)	Geometric mean of measured concentrations (mg/L)
1.00	0.0511
3.16	0.151
10.0	0.597
31.6	1.47
100	5.19

The value of geometric mean of the measured concentration is used in this following report.

4.1 Observation of test solution and measurement of water quality variables

(1) Appearance of test solution

At the start of the exposure, test solutions of the exposure levels were colorless and clear. At the end of the exposure, the appearance of test solution of 5.19 mg/L exposure level was light green and that of the other exposure levels were green due to the algae growth.

The solutions of the control level were colorless and clear at the start of the exposure, and those at the end of the exposure were green due to the algae growth.

(2) Water quality and environmental conditions

The measured values of pH in the test solutions were 8.0 at the start and 9.7 to 9.9 at the end of the exposure. The fluctuation of pH in control was out of the range of the regulation of the test method (not increase by more than 1.5 units in ordinary practice). Temperature in incubator ranged from 23.3 to 23.8°C and light intensity was 99 to 100 $\mu E/m^2/s$. The measured values of pH of test solution are shown in Table 1, and temperature and light intensity in the incubator are shown in Table 2.

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.0966 to 9.45 mg/L at the start of the exposure, 0.0436 to 5.02 mg/L at 24 hours after the start of the exposure, 0.0466 to 4.69 mg/L at 48 hours after the start of the exposure and 0.0445 to 3.73 mg/L at the end of the exposure. The percentages of the concentration at the start were 43.1 to 58.6% at 24 hours, 41.2 to 60.2% at 48 hours, and 39.4 to 51.1% at the end, respectively. The results of the measured concentrations of the test item are shown in Appendix 2.

4.2 EC_{50}

EC_{50} (E_rC_{50}) of the 13F-EtOH based on the growth rate was >5.19 mg/L. Values of cell concentration at each time, growth inhibition rates at exposure level and the EC_{50} are shown in Table 3, Table 4, and Table 5, respectively. Concentration-response curve is shown in Figure 1.

4.3 Growth curves in each test level, cell observations and NOEC

In the 5.19 mg/L exposure levels, the algae growth was logarithmic, although inhibition was observed. In the other exposure levels, the algae growths were close to control.

The following results of cell observation were based on the comparison with the control. In the 5.19 mg/L exposure level, a lot of distended cells were observed. In the other exposure levels, the condition of cells was the same as the control. The condition of cells was not abnormal in control.

By the results in statistical analysis and cell observation showed above, NOEC based on growth rate was 1.47 mg/L. NOEC, the result of statistical analysis of significant difference, and growth curve are shown in Table 5, Table 6 and Figure 2, respectively.

4.4 Validity of test

Detailed result on validity of the test is shown in Table 7.

(1) Growth of control

The cell in the control grew exponentially during the exposure. At the end of exposure, it increased to 58.4 or more times of the number of initial cells in the control. This meets the validity of the test: the cell growth in control should have increased by a factor of at least 16 times at 72 hours after the start of the exposure.

(2) The mean coefficient of variation for section-by-section specific growth rates in the controls

The mean coefficient of variation in the control was 19.4%. It meets the validity of the test: the mean coefficient of variation in the control must not exceed 35%.

(3) The coefficient of variation of average specific growth rates in replicate controls

The coefficients of variations were 2.44% in the control. They meet the validity of the test: the mean coefficient of variation in controls must not exceed 7%.

4.5 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at around the solubility of the test item in medium. As a result, EC_{50} (E_rC_{50}) was >5.19 mg/L and NOEC was 1.47 mg/L. The concentration of the test item in the test solution of the highest level at the start of exposure was low (9.45 mg/L) compared with the solubility in the medium (17.4 mg/L). However, considering the variation among test vessels for the measurement of the solubility (14.0-21.4 mg/L), it was judged that the concentration of test item at the preparation was around solubility in medium. The concentrations of test item in the test solutions were significantly decreased during the exposure. The reason was estimated to the limit in algae growth inhibition study of the volatile substance (volatilization to headspace in the test vessel). With regard to the environmental conditions of the test, increase in the unit of pH in control, which is out of the range of the regulation of the test method, was observed. It was decided that the increase of the unit of pH in control was due to the limitation of the algae growth inhibition study with volatile substance (gas exchange between outside and inside of the test vessel is impossible because of the test vessel of closed system). The environmental conditions except pH were within the suitable range. Therefore, it is concluded 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 pH of test solutions at start and end of exposure

Measured concentration*6 (mg/L)	pH	
	At the start	At the end
Control	8.0	9.7
0.0511	8.0	9.8
0.151	8.0	9.8
0.597	8.0	9.9
1.47	8.0	9.9
5.19	8.0	9.7

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

Table 2 Culture temperature and light intensity in incubator

Time	At the start	1-day	2-day	At the end
Culture temperature (°C)	23.3	23.8	23.8	23.8
Light intensity (μE/m^2/s)	100	100	99	100

Table 3 Value of cell concentration at each time

Measured concentration (mg/L)	No.	Cell concentration ($\times 10^4$ cells/mL)			
		0 hours*7	24 hours	48 hours	72 hours
Control	1	0.500	2.17	11.8	37.5
	2	0.500	2.23	11.0	36.1
	3	0.500	2.00	11.4	36.4
	4	0.500	2.47	10.6	29.2
	5	0.500	1.83	10.5	36.4
	6	0.500	2.12	12.3	39.7
	Mean	0.500	2.13	11.3	35.9
	S.D.	0	0.214	0.732	3.51
0.0511	1	0.500	2.14	13.0	40.4
	2	0.500	2.34	12.2	35.1
	3	0.500	2.12	10.4	29.1
	Mean	0.500	2.20	11.9	34.9
	S.D.	0	0.119	1.34	5.66
0.151	1	0.500	2.08	12.5	35.4
	2	0.500	2.37	13.5	33.2
	3	0.500	2.31	13.4	34.3
	Mean	0.500	2.25	13.1	34.3
	S.D.	0	0.157	0.510	1.12
0.597	1	0.500	1.93	11.9	30.2
	2	0.500	2.43	11.0	40.2
	3	0.500	2.38	11.0	33.0
	Mean	0.500	2.25	11.3	34.5
	S.D.	0	0.274	0.482	5.21
1.47	1	0.500	2.43	12.5	36.6
	2	0.500	2.05	12.8	31.9
	3	0.500	2.08	11.0	25.5
	Mean	0.500	2.19	12.1	31.3
	S.D.	0	0.211	0.941	5.59
5.19	1	0.500	1.74	7.96	24.4
	2	0.500	1.57	7.79	27.2
	3	0.500	1.34	7.63	25.2
	Mean	0.500	1.55	7.79	25.6
	S.D.	0	0.200	0.164	1.44

*7 The value based on the measured value of pre-culture

Table 4　Growth inhibition rates at exposure level

Measured concentration (mg/L)	No.	Growth rate (0-3d)	Inhibition rate (%)
Control	1	1.44	-
	2	1.43	-
	3	1.43	-
	4	1.36	-
	5	1.43	-
	6	1.46	-
	Mean	1.42	-
0.0511	1	1.46	-2.90
	2	1.42	0.420
	3	1.35	4.79
	Mean	1.41	0.771
0.151	1	1.42	0.192
	2	1.40	1.72
	3	1.41	0.971
	Mean	1.41	0.960
0.597	1	1.37	3.97
	2	1.46	-2.79
	3	1.40	1.89
	Mean	1.41	1.02
1.47	1	1.43	-0.567
	2	1.39	2.65
	3	1.31	7.93
	Mean	1.38	3.34
5.19	1	1.30	8.91
	2	1.33	6.37
	3	1.31	8.19
	Mean	1.31**	7.82

**:Significant difference ($p < 0.01$)

(Refer to Table 6 for the details of the statistics analysis results)

Table 5 EC_{50} and NOEC on growth rate

Endpoint	EC_{50} (mg/L)	NOEC (mg/L)
Growth rate	> 5.19	1.47

Table 6 Result of statistical analysis

Measured concentration (mg/L)	Endpoint
	Growth rate
0.0511	n.s.
0.151	n.s.
0.597	n.s.
1.47	n.s.
5.19	**
Statistical procedure	Bartlett's test One-way ANOVA Dunnett's multiple comparison test

**: Significant difference ($p < 0.01$)
n.s.: no significant difference

Table 7 Validity of test

< Variation for section-by-section specific growth rates in the controls >

Control No.	Mean	Standard deviation	Coefficient of variation (%)	
1	1.44	0.27	19.0	
2	1.43	0.22	15.2	
3	1.43	0.29	20.5	19.4
4	1.36	0.30	22.4	(Mean)
5	1.43	0.27	19.0	
6	1.46	0.30	20.5	

< Variation of average specific growth rates in replicate controls >

	0-3day
Mean	1.42
Standard deviation	0.03
Coefficient of variation (%)	2.44

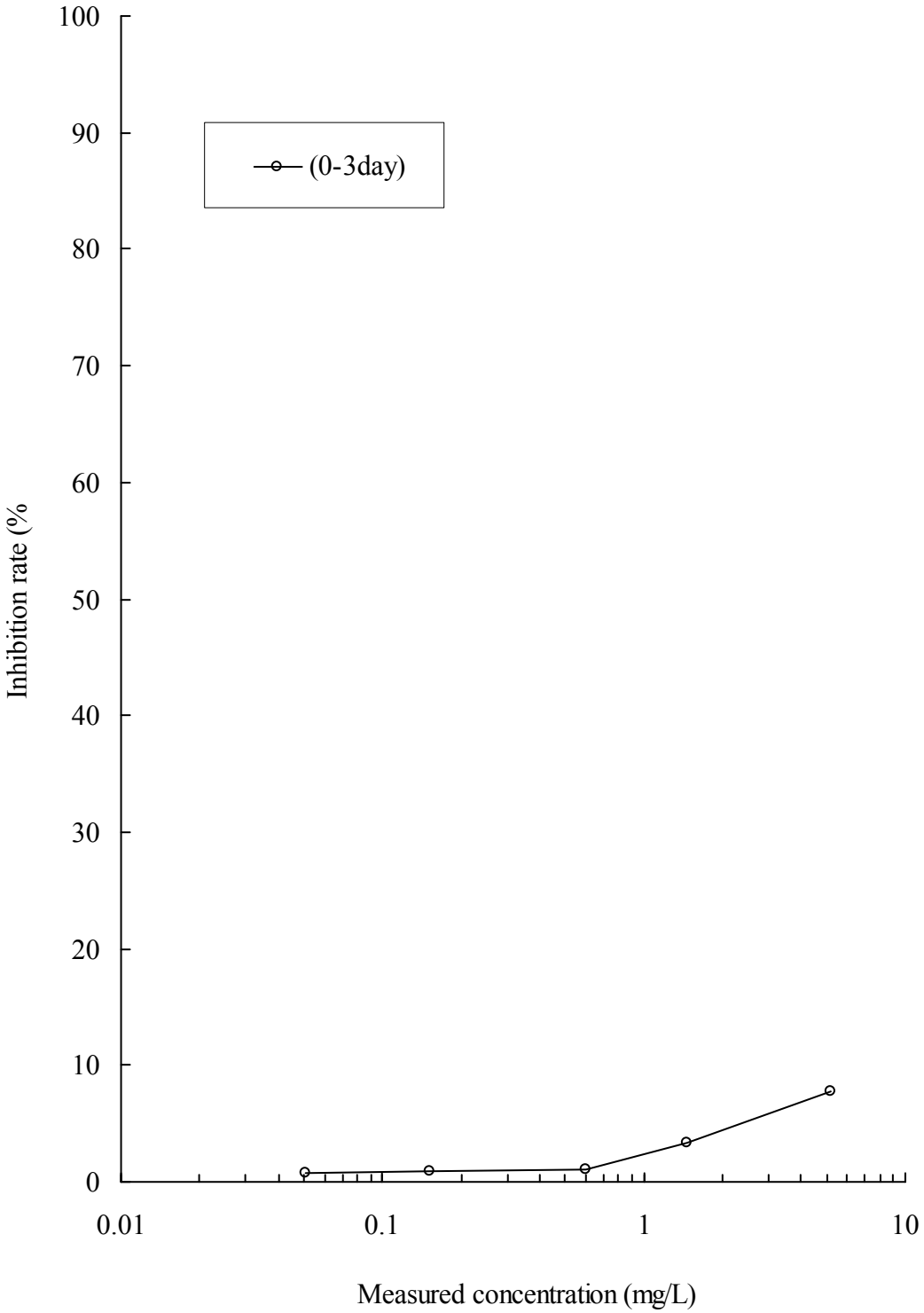


Figure 1 Concentration-response curve based on parameter of growth rate.

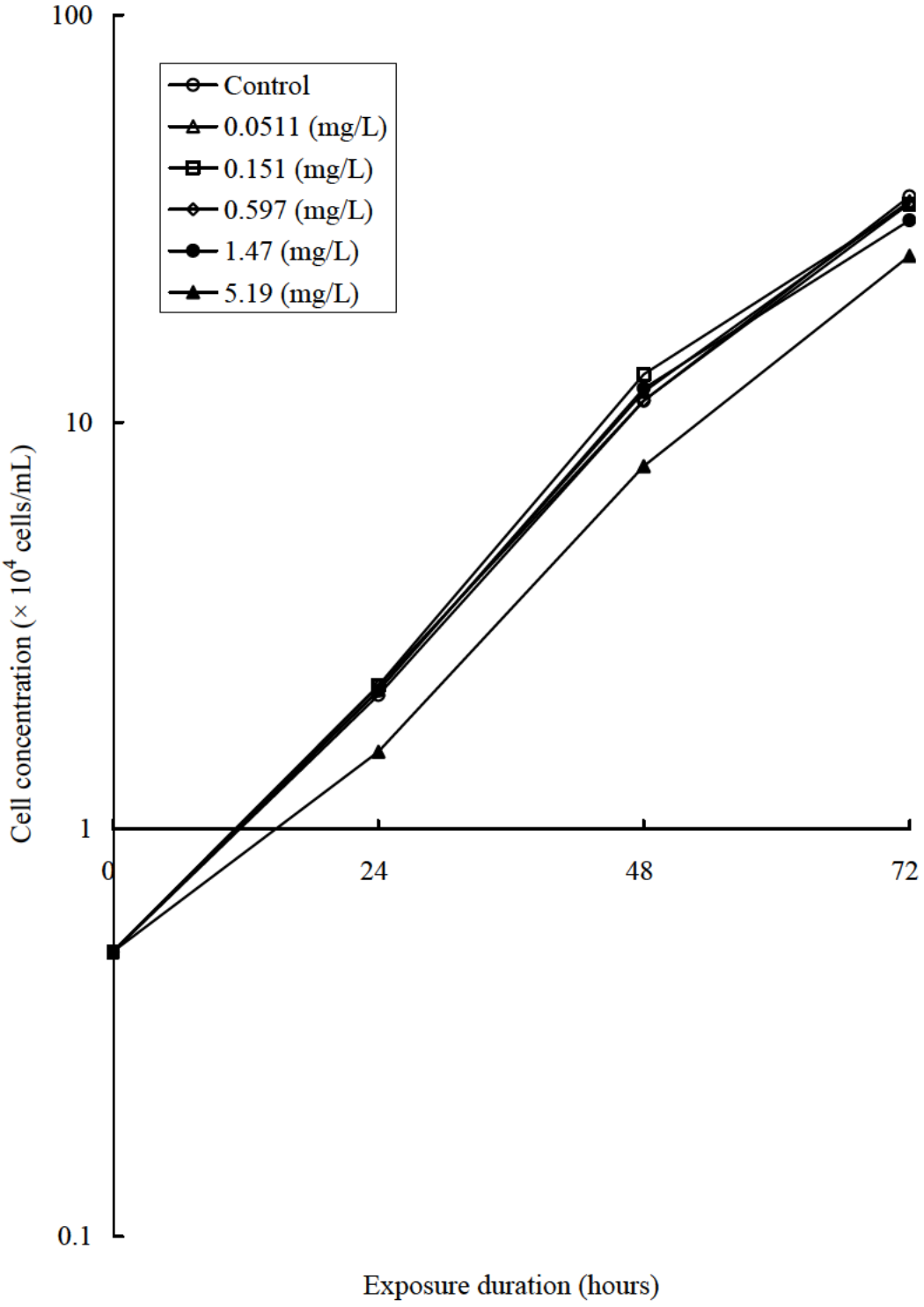


Figure 2 Growth curve in each test level.

Appendix 1

Composition of medium

Composition of OECD medium
[Guideline 201 (March 23, 2006)]

Nutrient salts	Amount	
H_3BO_3	0.185	mg
$MnCl_2 \cdot 4H_2O$	0.415	mg
$ZnCl_2$	0.003	mg
$FeCl_3 \cdot 6H_2O$	0.064	mg
$Na_2EDTA \cdot 2H_2O$	0.1	mg
$CoCl_2 \cdot 6H_2O$	0.0015	mg
$Na_2MoO_4 \cdot 2H_2O$	0.007	mg
$CuCl_2 \cdot 2H_2O$	0.00001	mg
$CaCl_2 \cdot 2H_2O$	18	mg
NH_4Cl	15	mg
KH_2PO_4	1.6	mg
$NaHCO_3$	50	mg
$MgCl_2 \cdot 6H_2O$	12	mg
$MgSO_4 \cdot 7H_2O$	15	mg

The constituents mentioned above were filled up to 1L with purified water.

Appendix 2

Analytical method and measured concentration of test item

1. Pretreatment of test solution

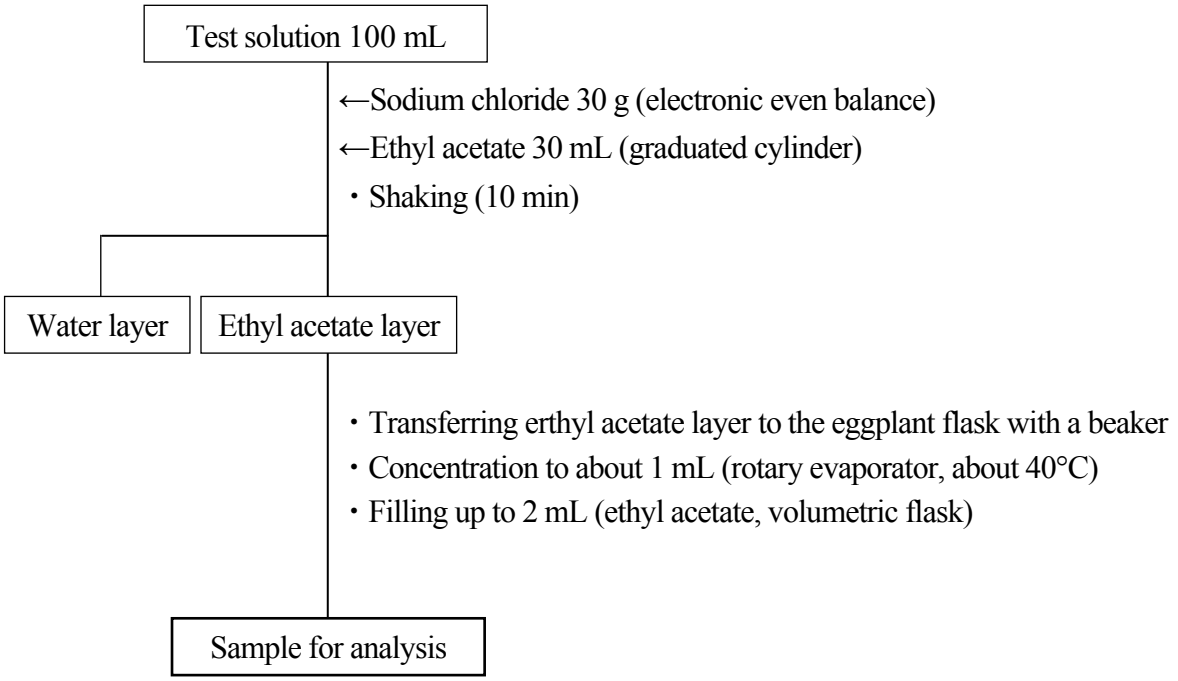
① 31.6 and 100%

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

② Control, 1.00, 3.16 and 10.0%

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 (31.6 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

② Analytical conditions (Control, 1.00, 3.16 and 10.0%)

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) $\xrightarrow{①}$ 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 31.6 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 / medium (1/1 v/v) to prepare 50.0 mg/L of test item solution. The solution was diluted with methanol / medium (1/1 v/v) to prepare 5.00 mg/L of standard solution.

② Control, 1.00, 3.16 and 10.0%

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 31.6 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.

② Control, 1.00, 3.16 and 10.0%

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 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 0.0123 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 medium according to pretreatment of test solution described in section 1 (Pretreatment of test solution ②). The blank test was also conducted using medium (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 pretreatmen

82.4%, 80.8% average 81.6%

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 the start %)				
	At the start	24 hours	48 hours	At the end	Geometric
Control	n.d.	n.d.	n.d.	n.d.	
1.00	0.0966	0.0436 (45.2)	0.0466 (48.2)	0.0445 (46.1)	0.0511
3.16	0.291	0.132 (45.4)	0.126 (43.2)	0.149 (51.1)	0.151
10.0	0.950	0.557 (58.6)	0.572 (60.2)	0.468 (49.2)	0.597
31.6	3.03	1.31 (43.1)	1.25 (41.2)	1.25 (41.2)	1.47
100	9.45	5.02 (53.1)	4.69 (49.6)	3.73 (39.4)	5.19

n.d. : <0.0123 mg/L

The geometric mean is calculated by the following expression:

$$\text{antilog}\left(\frac{1}{2(t_n - t_1)}\sum_{i=1}^{n-1}\left[\left(\log(conc_i) + \log(conc_{i+1})\right)\cdot(t_{i+1} - t_i)\right]\right)$$

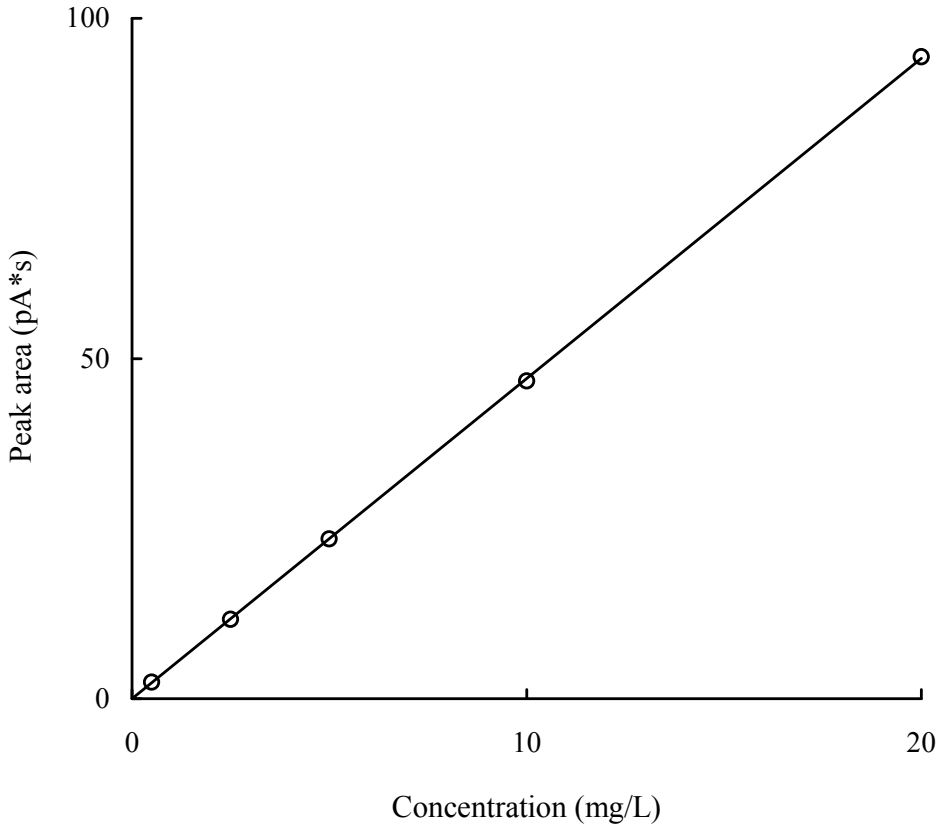
where

t_1 = at the start < t_2 < $\cdots$ t_n = at the end

$conc_1$ = concentration at the start, $conc_2$, $\cdots$, $conc_n$ = concentration at the end

Appendix 3

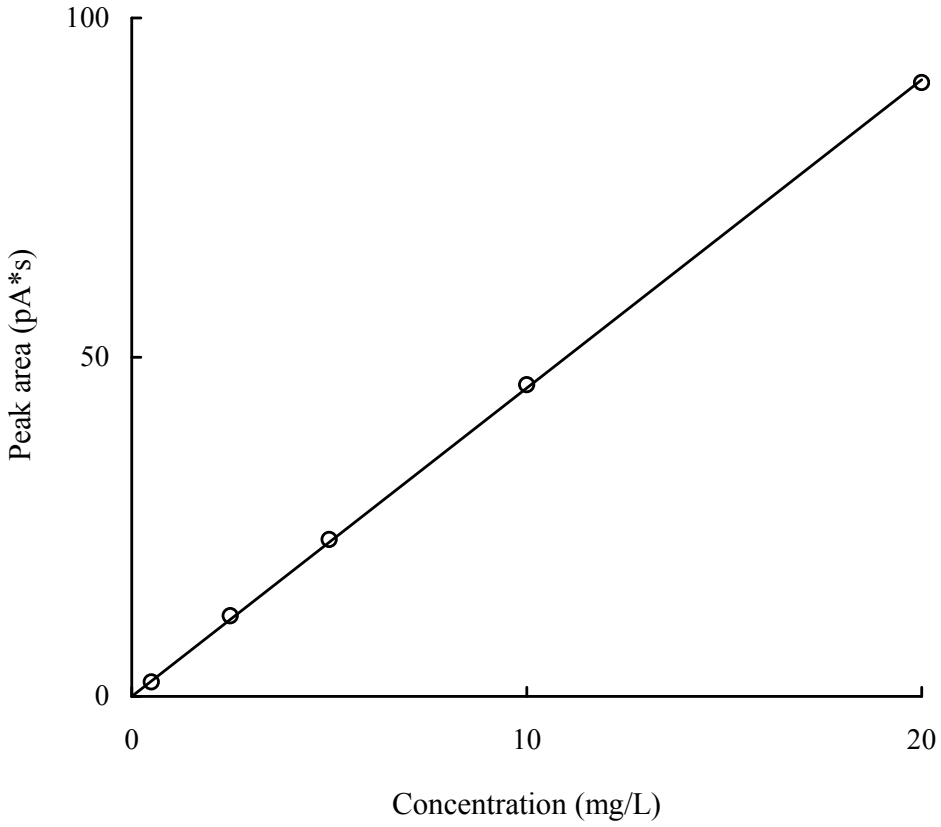
Calibration curve and chromatogram



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, 1.00, 3.16 and 10.0%).



y = 4.54x
r = 1.00

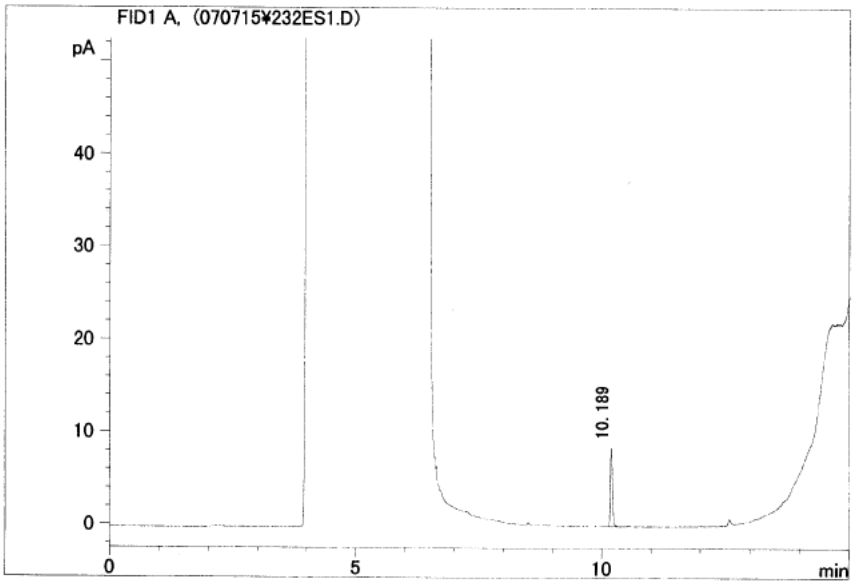
Concentration (mg/L)	Peak area (pA*s)
0.500	2.121
2.50	11.867
5.00	23.122
10.0	45.947
20.0	90.445

Appendix figure 3-1-2 Calibration curve of 13F-EtOH for analysis by GC(31.6 and 100%).

Standard solution 5.00 mg/L

Study No. 94232

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¥070715¥232ES1.D
サンプル名 : std 5.00mg/L



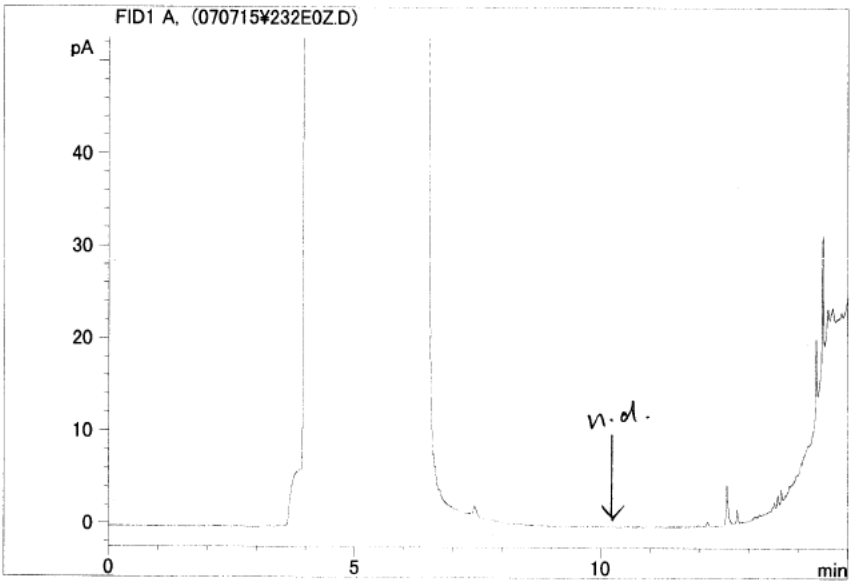
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	23.240	8.471	10.189

Appendix figure 3-2-1 GC chromatogram of standard solution at start of exposure (For control, 1.00, 3.16 and 10.0%).

Control

Study No. 94232

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¥070715¥232E0Z.D
サンプル名 : Cont(*50)H0h



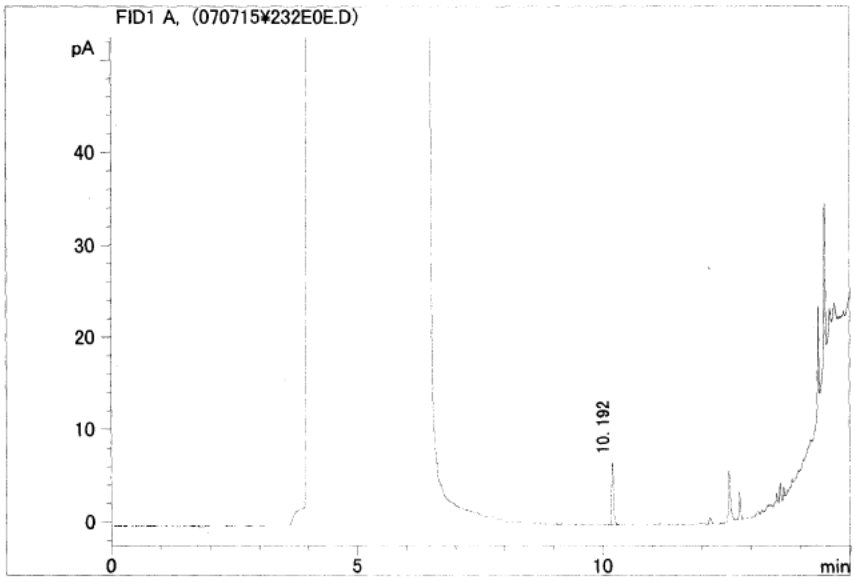
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

1.00% (Stock solution)

Study No. 94232

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¥070715¥232E0E.D
サンプル名 : 1.00%(*50)H0h



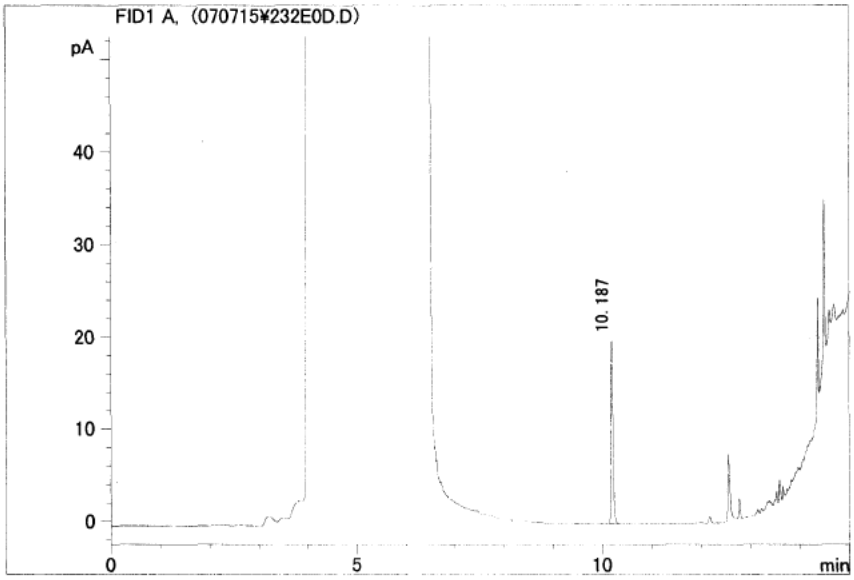
#	メインピーク面積	メインピーク高さ	実測 RT
1	18.328	6.680	10.192

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

3.16% (Stock solution)

Study No. 94232

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¥070715¥232E0D.D
サンプル名 : 3.16%(*50)H0h



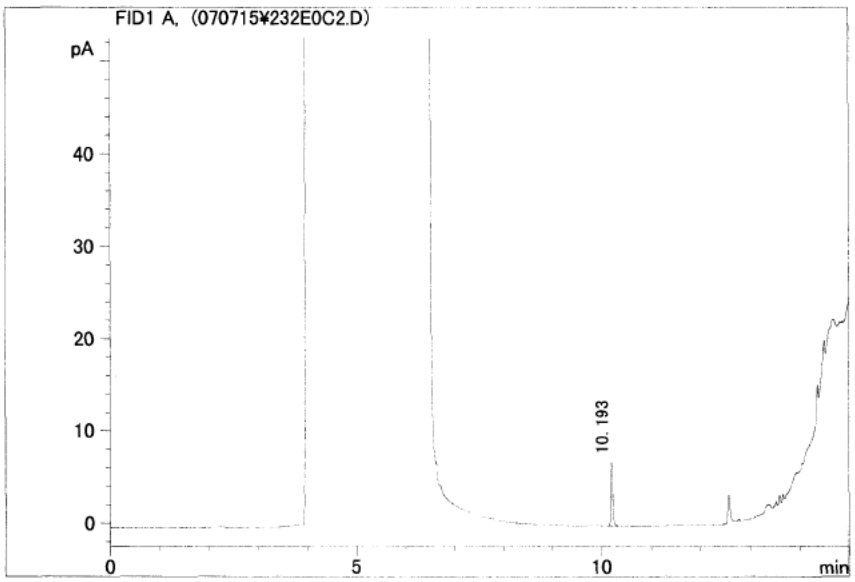
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	55.162	19.804	10.187

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

10.0% (Stock solution)

Study No. 94232

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¥070715¥232E0C2.D
サンプル名 : 10.0%(*50D10)H0h



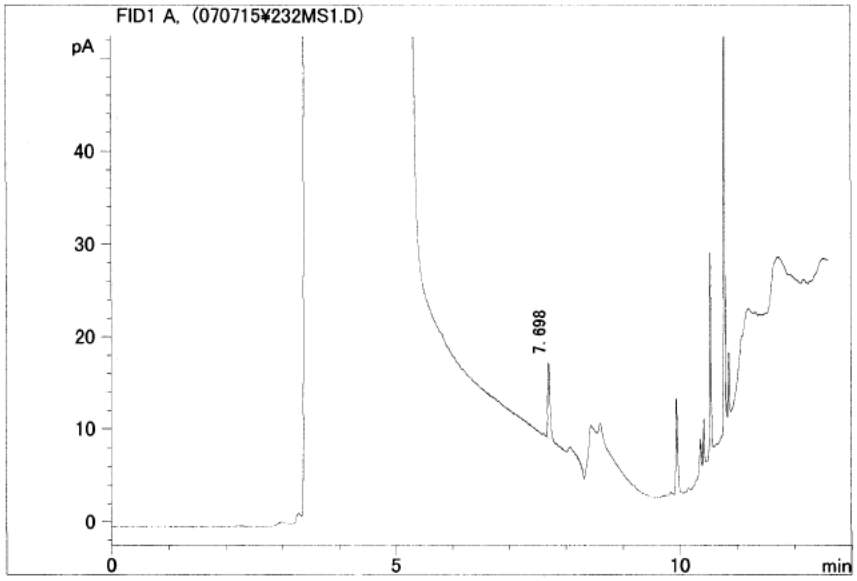
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	18.022	6.854	10.193

Appendix figure 3-2-5 GC chromatogram of test solution at start of exposure.

Standard solution 5.00mg/L

Study No. 94232

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¥070715¥232MS1.D
サンプル名 : std 5.00mg/L



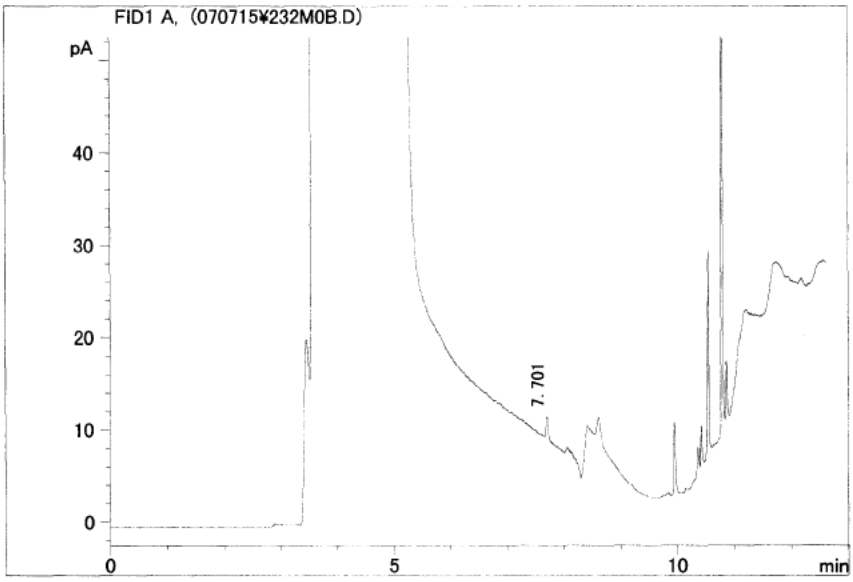
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	22.240	8.223	7.698

Appendix figure 3-2-6 GC chromatogram of standard solution at start of exposure (For 31.6 and 100%).

31.6% (Stock solution)

Study No. 94232

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¥070715¥232M0B.D
サンプル名 : 31.6%(D2)H0h



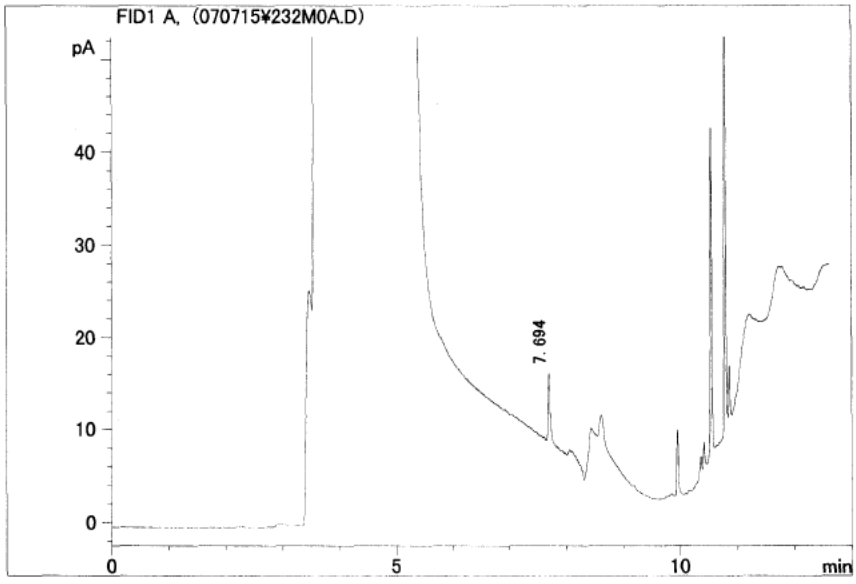
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	6.740	2.516	7.701

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

100% (Stock solution)

Study No. 94232

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¥070715¥232M0A.D
サンプル名 : 100%(D2)H0h



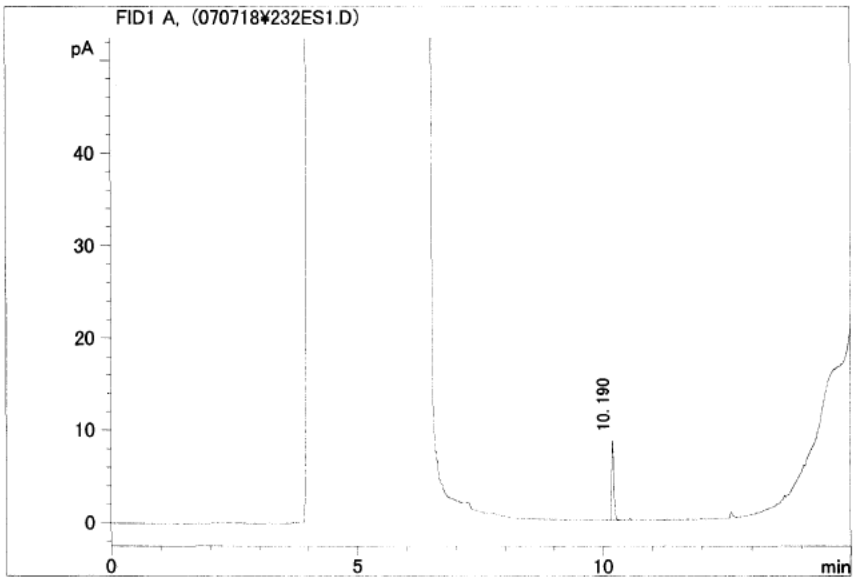
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	21.024	7.514	7.694

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

Standard solution 5.00 mg/L

Study No. 94232

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¥070718¥232ES1.D
サンプル名 : std 5.00mg/L



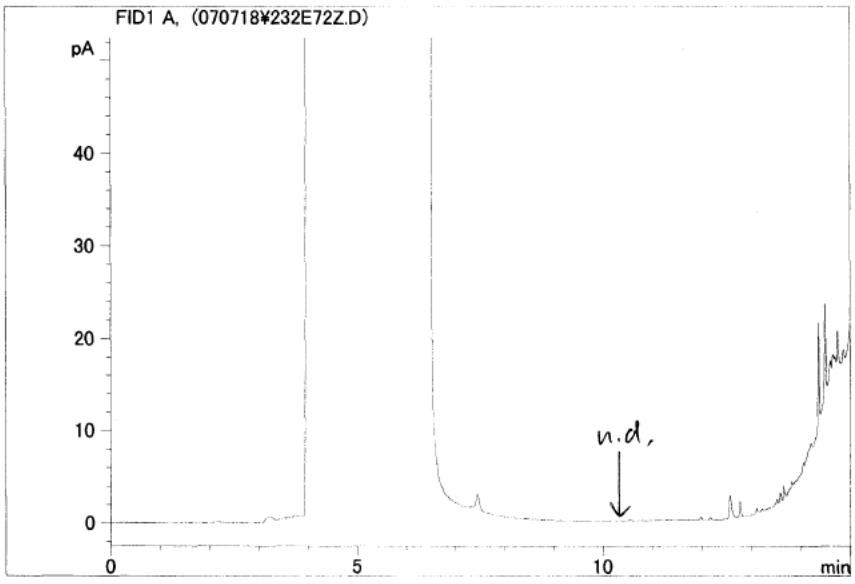
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	23.728	8.618	10.190

Appendix figure 3-3-1 GC chromatogram of standard solution at end of exposure (For control, 1.00, 3.16 and 10.0%).

Control

Study No. 94232

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¥070718¥232E72Z.D
サンプル名 : Cont(*50)H72h



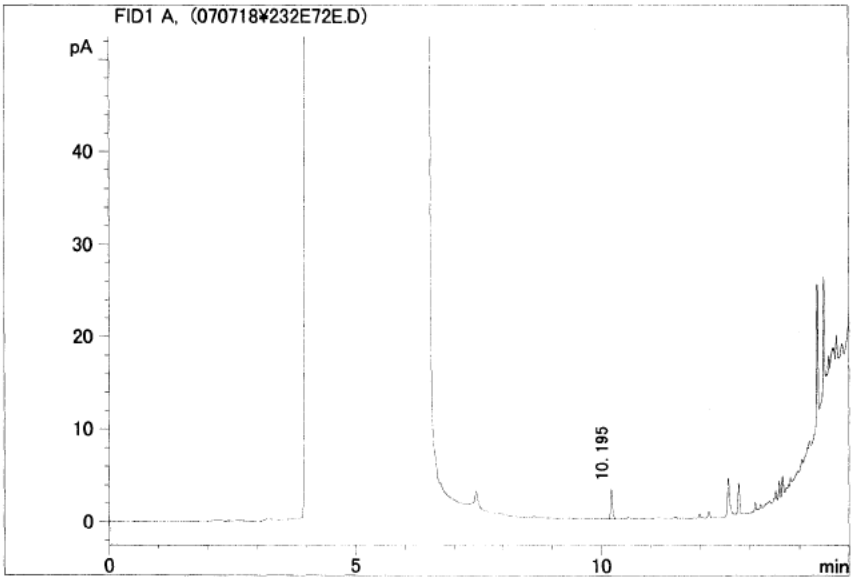
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT

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

1.00% (Stock solution)

Study No. 94232

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¥070718¥232E72E.D
サンプル名 : 1.00%(*50)H72h



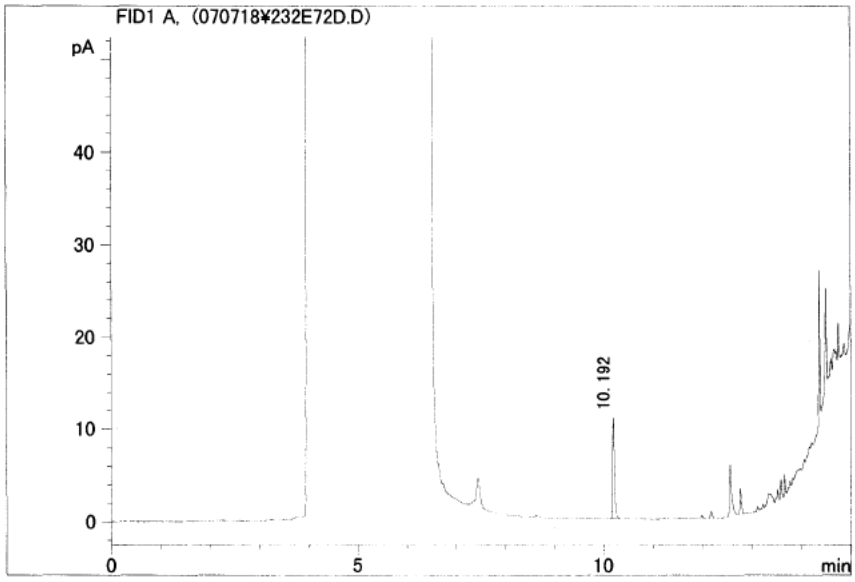
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	8.618	3.203	10.195

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

3.16% (Stock solution)

Study No. 94232

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¥070718¥232E72D.D
サンプル名 : 3.16%(*50)H72h



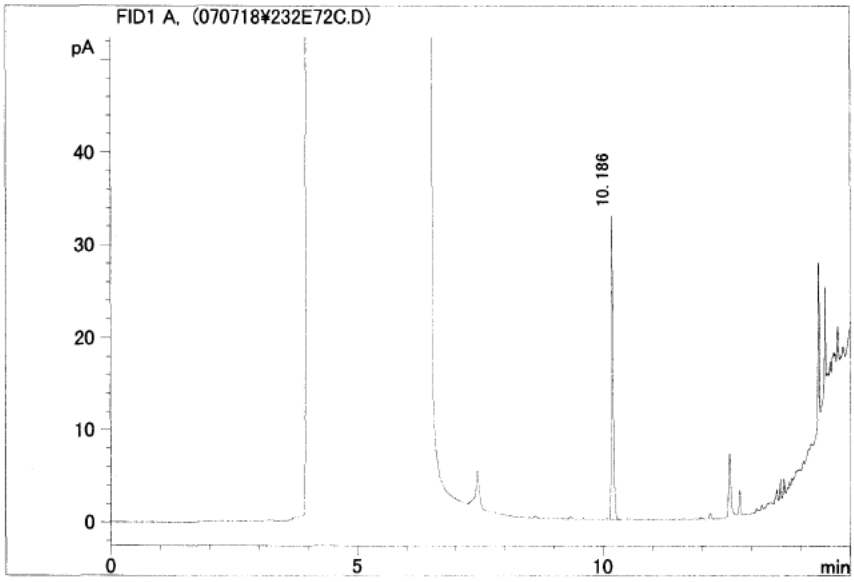
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	28.763	10.782	10.192

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

10.0% (Stock solution)

Study No. 94232

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¥070718¥232E72C.D
サンプル名 : 10.0%(*50)H72h



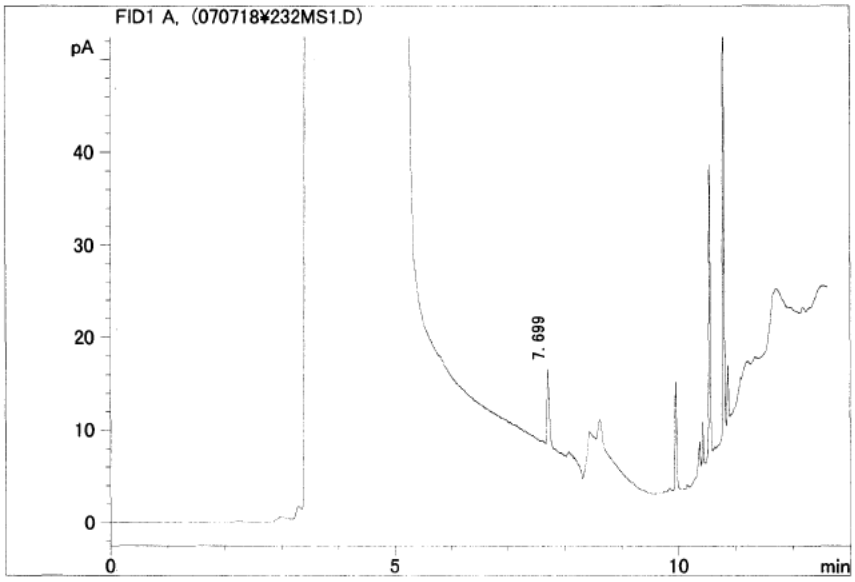
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	90.589	32.768	10.186

Appendix figure 3-3-5 GC chromatogram of test solution at end of exposure.

Standard solution 5.00 mg/L

Study No. 94232

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¥070718¥232MS1.D
サンプル名 : std 5.00mg/L



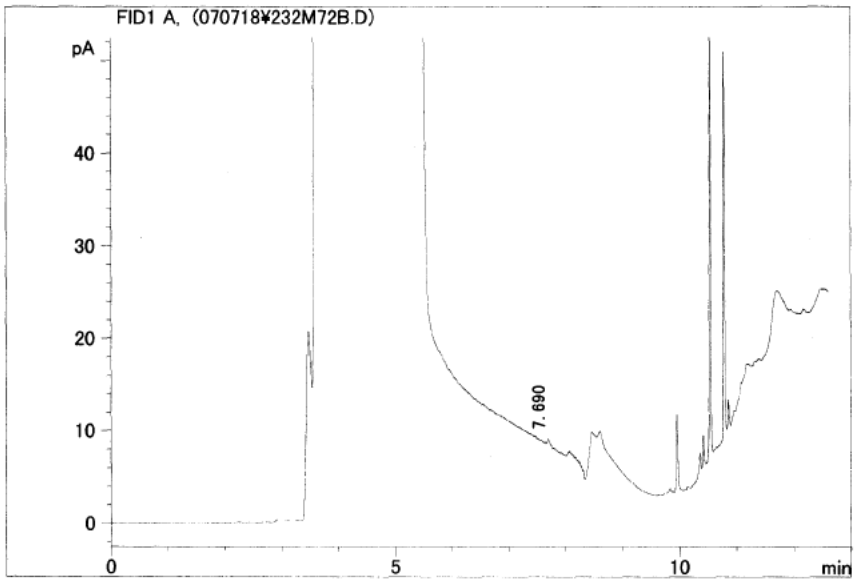
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	22.253	8.192	7.699

Appendix figure 3-3-6 GC chromatogram of standard solution at end of exposure (For 31.6 and 100%).

31.6% (Stock solution)

Study No. 94232

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¥070718¥232M72B.D
サンプル名 : 31.6%(D2)H72h



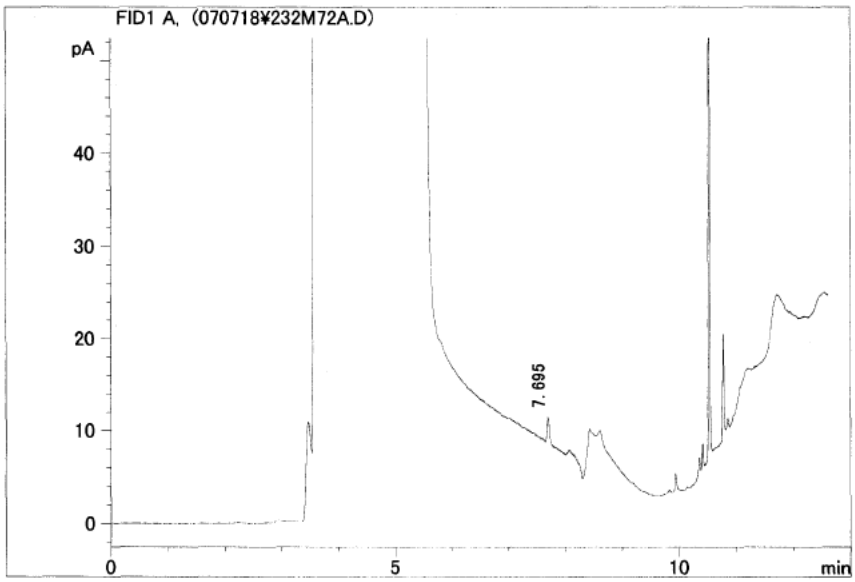
#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	2.778	0.763	7.690

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

100% (Stock solution)

Study No. 94232

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¥070718¥232M72A.D
サンプル名 : 100%(D2)H72h



#	ﾒｲﾝﾋﾟｰｸ面積	ﾒｲﾝﾋﾟｰｸ高さ	実測 RT
1	8.297	2.948	7.695

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

Appendix 4

Solubility in medium

1. Title

Solubility of test item in medium

2. Objective

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

3. Outline

Test item mixed with medium was stirred for 24 and 48 hours under the test temperature. After clearing insoluble matter, solubility was analyzed.

4. Performance of test

4.1 Test equipments and instruments

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

4.2 Test conditions

(1) Test temperature : 23±1°C

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

(3) 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 medium 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 container 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 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 / medium (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 medium. The solubility of the test item to medium was 17.4 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	18.6	19.4	15.8
	b	20.7		
	c	19.0		
Sample-2	a	13.5	13.5	
	b	13.3		
	c	13.7		
Sample-3	a	14.4	14.4	
	b	15.6		
	c	13.2		

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	16.6	16.8	17.4
	b	15.9		
	c	17.9		
Sample-5	a	21.4	20.1	
	b	20.5		
	c	18.5		
Sample-6	a	16.6	15.1	
	b	14.0		
	c	14.7		

*2 Arithmetic mean value

Additional data

Results of preliminary studies

1. Solubility of test item in medium

It was expected that the solubility of the test item in medium was below 100 mg/L, therefore, the measurement of the solubility of the test item in medium was conducted.

1) Preliminary study for measurement of solubility

(1) Method

Since the test item was expected to volatile due to the chemical structure, the test item and medium were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature (23±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	17.4	18.3
	②	19.1	
	③	18.4	
Sample-2	①	18.1	17.9
	②	17.1	
	③	18.5	

Value measured after stirring for 48 hours

Sample name		Measured value(mg/L)	Arithmetic mean value (mg/L)
Sample-3	①	19.1	20.6
	②	21.7	
	③	21.0	
Sample-4	①	19.7	19.3
	②	19.9	
	③	18.3	

Solubility of test item in medium was around 17 to 22 mg/L.

2) Summary of preliminary study for measurement of solubility

By the results of preliminary study for measurement of solubility, the solubility of the test item in medium was around 17 to 22 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, 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

The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration) as upper limit of test concentration. Then, they were stirred for approximately 48 hours. After the mixture was left at rest for about 1 hour, the saturated solution of test item was collected from the middle layer for use as the test solution. Then the test solution was prepared by diluting the stock solution with medium accordingly. The test solution was divided into each test vessel. The closed test vessel for volatile substance was used. The test organisms were exposed to the test solutions to confirm the effect.

(2) Result

Exposure level Content of stock solution (%)	Growth inhibition rate (%)
	Growth rate (0-3d)
1.00	-0.173
10.0	0.647
30.0	1.13
100	26.5

Replicates: one replicate / exposure level, two replicates / control level

Measurement method: cell counting method

In the 100% exposure level, approximately 27% of inhibition was confirmed. In the 1.00% exposure level, no effect was found.

2) Preliminary study 2

(1) Method

The test solution was prepared in the same manner as preliminary study 1 and was exposed to the test organisms to confirm the effect. The concentrations of the test item in the test solutions were measured simultaneously. The confirmation whether the algae took in the test item was not conducted because the separate operation such as centrifugation was impossible for volatile substance.

(2) Result

Exposure level Content of stock solution (%)	Growth inhibition rate (%)
	Growth rate (0-3d)
1.00	-4.09
3.00	-7.32
10.0	-8.99
30.0	-8.10
100	2.24

Replicates: two replicates / test level

Measurement method: cell counting method

In the 100% exposure level, approximately 2% of inhibition was confirmed.

Exposure level Content of stock solution (%)	Measured concentration (mg/L) (Percent of the measured concentration versus that at the start of the exposure %)	
	At the start of the exposure	At the end of the exposure
1.00	0.110	0.0649 (59.2)
100	12.7	6.68 (52.7)

The concentration of the test item at the start of the exposure was close to solubility in medium. The measured concentration of the test item significantly decreased at the end of the exposure, resulting in approximately 50% of that at the start of the exposure.

3) Summary of effect on test organisms (results of preliminary studies)

In 100% exposure level, 2 to 27% of inhibition was found. It was considered that no effect was confirmed around 1.00% exposure level. The concentration of the test item significantly decreased during the exposure because of such as volatilization of the test item. The exchange of the test solution was impossible in the algae growth inhibition study, and appropriate headspace in the test vessel was necessary for the growth of algae. Therefore, it was difficult to maintain the concentration of the saturated solution as a test design.

3. Operation of the definitive study

1) Measurement of solubility of test item in medium

Based on the results of the preliminary study for the solubility, measurement of the solubility of the test item in medium was conducted as follows. The test sample and medium were mixed to prepare the nominal concentration of 100 mg/L. Then, they were stirred gently for 24 and 48 hours at 23 ± 1°C (temperature of algae growth inhibition study) in the closed vessel. Removal of the insoluble matter such as the centrifugation and filtration was not conducted. After stirring, the mixed solution was left at rest for 1 hour and the test solution was collected from the middle layer of it in order to remove insoluble matter as possible. Then, the concentration of the test item in the test solution was measured.

2) Definitive study

Based on the results of the preliminary studies, the definitive study was conducted as follows. The test sample and medium were mixed to prepare approximately 100 mg/L (nominal concentration). Then, they were stirred for about 48 hours in the closed vessel with little headspace. After the mixture was left at rest for about 1 hour, the stock solution of test item (saturated solution) was collected from the middle layer. The solution was diluted with medium accordingly to prepare the test solution. The definitive study was conducted with five concentrations which were 100, 31.6, 10.0, 3.16 and 1.00% of stock solution (a geometric series with a factor of $\sqrt{10}$). The closed test vessel for volatile substance was used. It was expected that the concentrations of the test item in the test solutions were decreased during the exposure. Therefore, the concentrations of the test item in the test solutions were measured at the start of the exposure, 24 and 48 hours after the exposure, and the end of the exposure.