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FINAL REPORT

Biodegradation study of (CBI) by microorganisms

September 18, 2003

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

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Company Sanitized

MR 297333

[redacted]

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14114

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No.14114, issued on September 18, 2003, amended on October 10, 2003).

Date December 2, 2003

Study Director Y. Matsunobu
Yasuko Matsunobu

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD

Title Biodegradation study of (CBI) by microorganisms

Study number 14114

This study was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

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

Date September 18, 2003

Study Director Signed in original

Yasuko Matsunobu

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14114

Amendment to the Final Report was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997)

This GLP statement was issued as supplement to the statement issued on September 18, 2003 because of the amendment of the Final Report.

Date October 10, 2003

Study Director Signed in original

Yasuko Matsunobu

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14114

The inspections of this study were carried out and the results were reported to the test facility management and the Study Director by Quality Assurance Unit of Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan as follows.

Item of inspection	Date of inspection	Date of report to Study Director	Date of report to test facility management
Study plan	July 17, 2003	July 18, 2003	July 18, 2003
	August 11, 2003	August 11, 2003	August 11, 2003
	September 10, 2003	September 10, 2003	September 10, 2003
Test Conduct	July 18, 2003	July 18, 2003	July 18, 2003
	August 1, 2003	August 26, 2003	August 26, 2003
	August 15, 2003	August 26, 2003	August 26, 2003
	August 18, 2003	August 26, 2003	August 26, 2003
	August 21, 2003	August 26, 2003	August 26, 2003
	August 25, 2003	August 26, 2003	August 26, 2003
	August 26, 2003	August 26, 2003	August 26, 2003
Raw Data and Final Report	September 18, 2003	September 18, 2003	September 18, 2003

It has been assured that the final report describes accurately the test method used, that details in the report are in compliance with the study plan and Standard Operating Procedures and that the final report reflects the raw data accurately.

Date September 18, 2003

Quality Assurance Unit, Head Signed in original

Kyoshiro Hori

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14114

Study inspection of the corrected parts in the Final Report was carried out and it was confirmed that the correction has no problem. The result was reported to the test facility management and the Study Director as follows.

Date of inspection	Date of report to Study Director	Date of report to test facility management
October 10, 2003	October 10, 2003	October 10, 2003

This statement was issued as a supplement to the quality assurance statement issued on September 18, 2003.

Date October 10, 2003

Quality Assurance Unit, Head Signed in original

Kyoshiro Hori

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Study No 14114

Title

Biodegradation study of (CBI) by microorganisms

Sponsor

DAIKIN INDUSTRIES, LTD.
1-1 Nishi-hitotsuya, Settsu, Osaka 566-8585, Japan

Test facility

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan
19-14 Chuomachi, Kurume, Fukuoka 830-0023, Japan

Objective

This study was performed to evaluate the biodegradability of (CBI) by microorganisms.

Test method

This study was performed according to "Ready Biodegradability: Modified MITI Test (I) (Guideline 301C, Revised July 17, 1992)" in the OECD Guideline for Testing of Chemicals.

Applied GLP

This study was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

Dates

Study initiation date	July 17, 2003
Experimental starting date	July 18, 2003
Experimental completion date	August 15, 2003
Study completion date	September 18, 2003

Storage of test item, raw data, etc.

(1) Test item

About 5 g of the item supplied by the sponsor is sealed in a store vessel and stored in archives in this laboratory for ten years after the publication of the final report. Treatment of the item supplied by the sponsor after the storage period is discussed with sponsor. If it is not stable for the storage period, it is stored while it is kept stable and it is disposed with approval of sponsor.

(2) Raw data and materials, etc.

Raw data, the study plan, documents about the study presented by the sponsor, the final report and necessary materials are stored in archives in this laboratory for the same term as the test item. Treatment of raw data and materials, etc. after the storage period is discussed with sponsor.

Personnel

Study Director	Yasuko Matsunobu
Study personnel (Operation of biodegradation test)	Kazuhiro Oyama Takakazu Kayashima
Staff for cultivation of activated sludge	Hiroto Nishijima

Approval of final report

Study Director	Date	September 18, 2003
	Signature	Signed in original
		Yasuko Matsunobu

SUMMARY

Title

Biodegradation study of (CBI) by microorganisms

Conditions of cultivation

(1) Concentration of test item	100 mg/L
(2) Concentration of activated sludge (as the concentration of suspended solid)	30 mg/L
(3) Volume of test solution	300 mL
(4) Cultivation temperature	25±1 °C
(5) Cultivation duration	28 days

Measurement and analysis for percentage biodegradation

(1) Measurement of biochemical oxygen demand (BOD) by means of a closed system oxygen consumption measuring apparatus
(2) Determination of test item by means of weight measurement

Other measurement and analysis

(1) Determination of dissolved organic carbon by means of total organic carbon (TOC) analysis
(2) Measurement of IR spectrum by means of a fourier transform infrared spectrophotometer
(3) Determination of perfluorooctanoic acid by means of liquid chromatography-mass spectrometry
(4) Determination of 2-(perfluorooctyl)ethanol by means of gas chromatography-mass spectrometry

Results

(1) Percentage biodegradation by BOD	2 %,	4 %,	6 %	average 4 %
(2) Percentage biodegradation by weight measurement	0 %,	0 %,	0 %	average 0 %

Conclusion

The test item was not biodegraded by microorganisms under the present test conditions.

1. Test item

In this report, (CBI) has the following chemical name, etc.

1.1 Chemical name*1

(CBI)

1.2 Chemical structure, etc.*1

Structural formula

Molecular weight Weight-average (CBI)

*1 Information supplied by the sponsor

2 Item supplied by sponsor

2.1 Supplier and lot number*1

(1) Supplier DAIKIN INDUSTRIES, LTD.

(2) Lot number (CBI)

2.2 Purity*1

(1) Test item (CBI)

(2) Impurity (CBI)

The test item was treated as 100 % in purity.

2.3 Confirmation of test item

Two infrared (IR) spectra of the test item provided by the sponsor and measured at this laboratory were confirmed to be identical (see Fig 10 and Reference).

2.4 Physicochemical property*1

Appearance (CBI)

*1 Information supplied by the sponsor

2.5 Storage and stability

(1) Storage condition

Dark storage place at room temperature

(2) Stability

The test item was stable under the storage conditions, as shown by the finding that IR spectra of the test item before the experimental start and after the experimental completion were identical (see Fig.10).

3. Activated sludge

3.1 Sludge sampling sites and date

(1) Sampling sites

On-site sludge sampling was carried out at the following 10 locations in Japan.

Fushikogawa city sewage plant (Sapporo-shi, Hokkaido)
Fukashiba industrial sewage plant (Kashima-gun, Ibaraki)
Nakahama city sewage plant (Osaka-shi, Osaka)
Ochiai city sewage plant (Shinjuku-ku, Tokyo)
Kitakami River (Ishinomaki-shi, Miyagi)
Shinano River (Niigata-shi, Niigata)
Yoshino River (Tokushima-shi, Tokushima)
Lake Biwa (Otsu-shi, Shiga)
Hiroshima Bay (Hiroshima-shi, Hiroshima)
Dookai Bay (Kitakyushu-shi, Fukuoka)

(2) Date June, 2003

3.2 Sludge sampling

(1) City sewage

Return sludges from sewage plants were collected.

(2) Rivers, lake and sea

Surface water and surface soil which was in contact with the atmosphere were collected.

3.3 Preparation of activated sludge

Activated sludge was prepared as follows to maintain its uniformity.

The filtrate (5 L) of the supernatant of the activated sludge*2 cultivated about for 3 months was mixed with the mixed filtrate (5 L) of the supernatant of a sludge collected newly at each location. The mixed filtrate (10 L) was aerated*3 after the pH value of the mixture was adjusted to 7.0±1.0.

*2 The activated sludge cultivated the mixed filtrate (10 L) of the supernatant of sludge collected at the ten locations.

*3 Prefiltered open air was used.

3.4 Cultivation

Roughly 30 minutes after ceasing aeration of the sludge mixture, supernatant corresponding to about 1/3 of the whole volume was removed. Dechlorinated water was added to the remaining portion so that the total volume reached 10 L. This mixture was aerated, and then a predetermined amount of synthetic sewage*4 was added to the mixture so that the concentration of the synthetic sewage was 0.1 wt% in the volume of dechlorinated water added. This procedure was repeated once every day. Cultivation was carried out at 25±2 °C.

*4 Synthetic sewage

Glucose, peptone and potassium dihydrogenphosphate were dissolved in purified water to obtain 50 g/L of the solution for each component. The pH of the solution was adjusted to 7.0±1.0 with sodium hydroxide.

3.5 Control and use

During cultivation, the appearance of the supernatant, sedimentation of the sludge, formation of flock, pH, dissolved oxygen concentration in the solution and temperature were checked to maintain a normal state of sludge. It was confirmed that these were within the scope of the control standard stipulated in the "Testing Methods for New Chemical Substances", and these results were stored as raw data. Microflora in the activated sludge was microscopically observed and sludge with no abnormal symptoms was used for the test.

3.6 Inspection of activity and date of initiation of use of activated sludge

(1) Inspection of activity

Activity of the sludge was assessed using a reference item.

(2) Date of initiation of use July 15, 2003

4. Performance of biodegradation test

4.1 Preparations for test

(1) Measurement of concentration of suspended solid

The concentration of suspended solid was measured to determine the amount of activated sludge to add.

Method	In accordance with Japanese Industrial Standards (JIS) K 0102-1998 section 14.1
Date	July 15, 2003
Result	Concentration of suspended solid in the activated sludge was 3200 mg/L.

(2) Preparation of basal culture medium

Each 3 mL of solutions (a), (b), (c) and (d) shown below were made up to 1L with purified water.

The following stock solutions were prepared by use of analytical grade regents:

(a) Potassium dihydrogenphosphate, KH_2PO_4 8.50 g
Dipotassium hydrogenphosphate, K_2HPO_4 21.75 g
Disodium hydrogenphosphate dodecahydrate, $Na_2HPO_4 \cdot 12H_2O$ 44.60 g
Ammonium chloride, NH_4Cl 1.70 g
Dissolved in water and filled up to 1 L

(b) Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$ 22.50 g
Dissolved in water and filled up to 1 L

(c) Calcium chloride anhydrous, $CaCl_2$ 27.50 g
Dissolved in water and filled up to 1 L

(d) Iron(III) chloride hexahydrate, $FeCl3 \cdot 6H_2O$ 0.25 g
Dissolved in water and filled up to 1 L

(3) Reference item

Aniline (reagent grade, Showa Chemicals Inc. Lot No. HO-2729D) was used as a reference item to confirm that the sludge was sufficiently active.

This test is effective when the percentage biodegradation calculated from the BOD of aniline after 7 and 14 days exceeds 40 % and 65 %, respectively, according to regulation of the OECD Guideline for Testing of Chemicals.

4.2 Preparation of test solutions

Test solutions for analysis of the test item and perfluorooctanoic acid and test solutions for analysis of 2-(perfluorooctyl)ethanol were prepared, because the test item and converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected to form in test solutions, could not be analyzed by the same pretreatment.

Eleven test vessels were prepared. The following test solutions were prepared and cultured under the conditions described in section 4.3.

(1) Addition of test item or aniline

(a) Test solution (water + test item)

(n=2: Vessel No. 6 and a test solution for analysis of 2-(perfluorooctyl)ethanol)

In one test vessel, 30 mg of the item supplied by the sponsor was accurately weighed and added to 300 mL of purified water, so that the concentration of the test item reached 100 mg/L.

(b) Test solution (sludge + test item)

(n=6: Vessel No.1, 2, 3 and three of test solutions for analysis of 2-(perfluorooctyl)ethanol)

In each test vessel, 30 mg of the item supplied by the sponsor was accurately weighed and added to the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated), so that the concentration of the test item reached 100 mg/L.

(c) Test solution (sludge + aniline) (n=1: Vessel No.4)

In one test vessel, 29.5 μL [30 mg = 29.5 μL × 1.022 g/cm^3 (density)] of aniline was added into the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated), so that the concentration reached 100 mg/L.

(d) Test solution (control blank)

(n=2: Vessel No.5 and a test solution for analysis of 2-(perfluorooctyl)ethanol)

In one test vessel, nothing was added to the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated).

(2) Inoculation of activated sludge

The activated sludge cultivated under the conditions described in section 3 was added to each test vessel, (b), (c) and (d), so that the concentration of the suspended solid reached 30 mg/L.

4 3 Instruments and conditions of cultivation

(1) Instruments for cultivation

(a) Test solution for analysis of test item and perfluorooctanoic acid

Closed system oxygen consumption measuring apparatus

Temperature controlled bath and measuring unit :
Ohkura Electric Co., Ltd.

Data sampler : Asahi Techneion Co., Ltd.

Vessel 300 mL in volume (improved type)

Absorbent for carbon dioxide

Soda lime No.1 (for absorption of carbon dioxide,
Wako Pure Chemical Industries, Ltd.)

(b) Test solution for analysis of 2-(perfluorooctyl)ethanol

Closed system oxygen consumption measuring apparatus

Temperature controlled bath and measuring unit
(Only the temperature controlled bath was used) :
Ohkura Electric Co., Ltd.

Vessel 300 mL in volume (sealed improved type)

(2) Conditions of cultivation

Cultivation temperature	25±1 °C
Cultivation duration	28 days
Stirring method	Each test solution was stirred by a magnetic stirrer.

(3) Room Apparatus room No.511

4 4 Observation and measurement of test conditions

(1) Observation of test solution

During the cultivation period, the appearance of the test solution was observed periodically and conditions of the instruments were checked properly.

(2) Measurement of biochemical oxygen demand (BOD)

During the cultivation period, the change in BOD of the test solutions was measured by autorecording using a data sampler. Cultivation temperature was measured and recorded once a day. BOD of the test solutions for analysis of 2-(perfluorooctyl)ethanol was not measured.

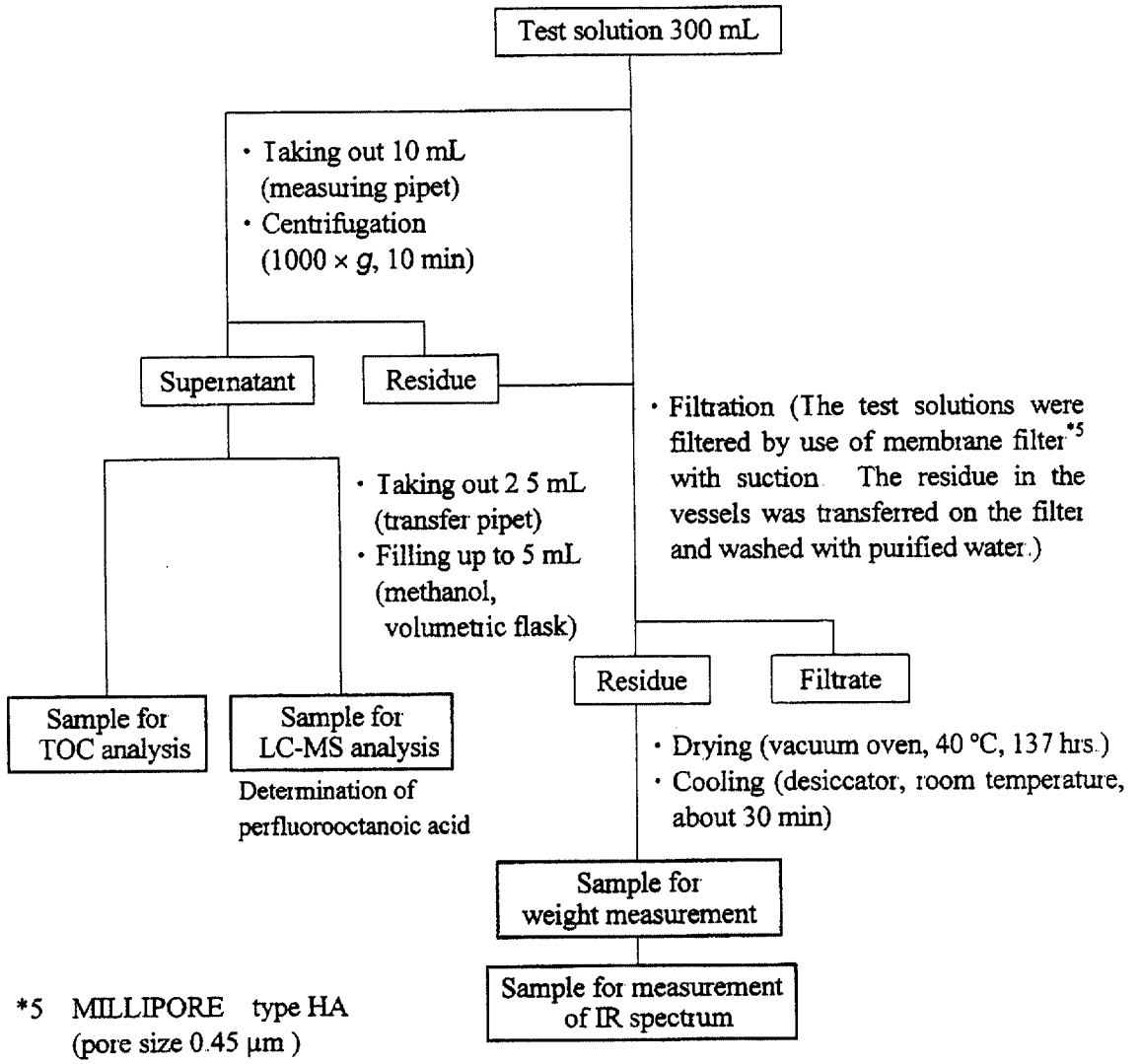
4.5 Analysis of test solution

After the termination of the cultivation, dissolved organic carbon, the test item and converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected production in test solutions, were determined.

4.5.1 Pretreatment of test solutions for analysis

(1) Pretreatment for analysis of test item and perfluorooctanoic acid

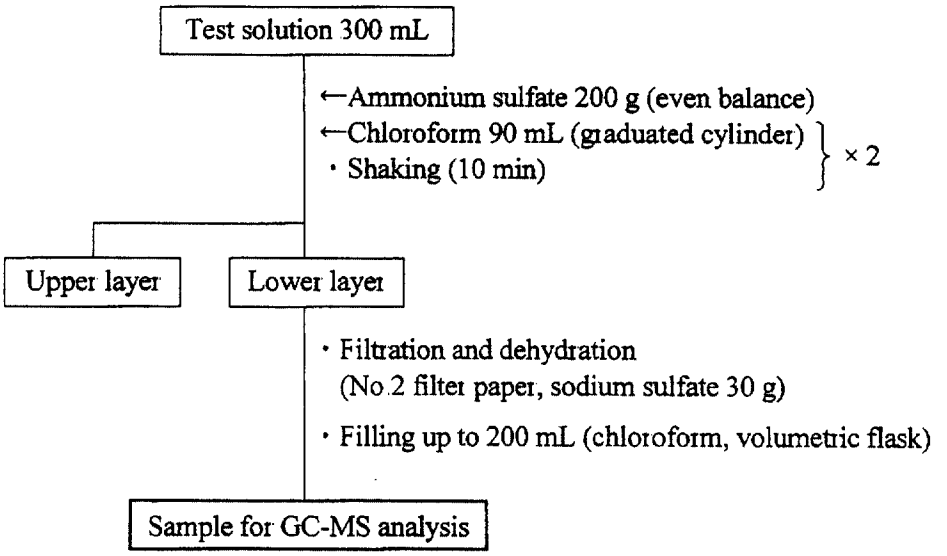
After the termination of the cultivation, the test solution (water + test item), the test solutions (sludge + test item) and the test solution (control blank) for analysis of the test item and perfluorooctanoic acid were pretreated for total organic carbon (TOC) analysis on dissolved organic carbon and liquid chromatography - mass spectrometry (LC-MS) analysis of perfluorooctanoic acid as follows. Weight measurement of the test item was performed. IR spectrum of the residue was measured.



*5 MILLIPORE type HA (pore size 0.45 μm)

(2) Pretreatment for analysis of 2-(perfluorooctyl)ethanol

After the termination of the cultivation, the test solution (water + test item), the test solutions (sludge + test item) and the test solution (control blank) for analysis of 2-(perfluorooctyl)ethanol were cooled at 5 °C for 66 hrs. and pretreated for gas chromatography - mass spectrometry (GC-MS) analysis as follows.



4.5.2 Quantitative and qualitative analysis

(1) Determination of dissolved organic carbon by means of TOC analysis

The dissolved organic carbon (DOC) in the samples for TOC analysis was analyzed under the following conditions.

The concentration of DOC was calculated by subtracting concentration of the inorganic carbon (IC) from concentration of the total carbon (TC). The concentration of TC and IC in the test solutions was proportionally calculated from the peak area of the test solution by comparison with that of 80.0 mgC/L of a standard solution for TC analysis and 80.0 mgC/L of a standard solution for IC analysis, respectively (see Table-2).

The standard solution for TC analysis was prepared by dissolving potassium hydrogenphthalate in purified water. The standard solution for IC analysis was prepared by dissolving sodium hydrogencarbonate and sodium carbonate in purified water.

The concentration of dissolved organic carbon corresponding to the minimum determination limit was regarded as 1.0 mgC/L.

Analytical conditions

Instrument	Total organic carbon analyzer Shimadzu Corporation type TOC-5000
Temperature of furnace	680 °C
Flow rate	150 mL/min
Injection volume	33 μL
Sensitivity	Range 5

(2) Determination of test item by means of weight measurement

Weight of the samples for weight measurement was measured under the following conditions (see Table-3).

Analytical conditions

Instrument	Electronic analytical balance Mettler Toledo type AT201
Minimum measuring weight	0.1 mg

(3) Measurement of IR spectrum by means of fourier transform infrared spectrophotometer

IR spectrum of the samples for measurement of IR spectrum was measured under the following conditions (see Fig.2).

Analytical conditions

Instrument	Fourier transform infrared spectrophotometer Shimadzu Corporation type FTIR-8200PC
Measuring method	KBr tablet

(4) Determination of perfluorooctanoic acid by means of LC-MS

The samples for LC-MS analysis were analyzed for perfluorooctanoic acid under the following conditions. The concentration of perfluorooctanoic acid in the sample for LC-MS analysis was proportionally calculated by comparing the peak area on the mass fragmentogram of the sample for LC-MS analysis with that on the mass fragmentogram of 0.500 mg/L standard solution (see Table-4 and Fig 6).

The lowest detectable peak area was regarded as 2000 considering the noise level, which corresponded to 0 0098 mg/L of perfluorooctanoic acid.

(a) Analytical conditions

Instrument	Liquid chromatograph–mass spectrometer
LC	JASCO Corporation type PU-980
MS	Micromass type Quattro II

Conditions of LC

Column	L-column ODS 15 cm × 4.6 mm I.D.
Eluent	Methanol[*6] / purified water[*6] (80/20 V/V)
Flow rate	1.0 mL/min
Sample size	10 μL

Conditions of MS

Ionization method	Electrospray (ESI)
Detection mode	Negative ion
Monitoring method	Selected ion monitoring (SIM)
Monitoring m/z	413 (see Fig.8)
Source temperature	150 °C
Cone voltage	20 V

*6 Containing 0.1 % formic acid

(b) Preparation of standard solution

The standard solution to determine the concentration of perfluorooctanoic acid in the sample for LC-MS analysis was prepared as follows.

100 mg of the standard substance*7 supplied by the sponsor was accurately weighed and dissolved in methanol to obtain 2000 mg/L solution of perfluorooctanoic acid. 0.500 mg/L standard solution was then prepared from this solution by dilution with methanol / purified water (1/1 V/V).

*7 Name, lot number and purity

Name	Perfluorooctanoic acid (C-1700)
Lot number	C17001401
Purity	Perfluorooctanoic acid was treated as 100 % in purity

(c) Calibration curve

0.125, 0.250 and 0.500 mg/L standard solutions were prepared by the same method as described in (b). These solutions were analyzed according to the analytical conditions described in (a). A calibration curve was drawn based on the relation between the peak area on the mass fragmentograms and the respective concentrations (see Fig.3).

(5) Determination of 2-(perfluorooctyl)ethanol by means of GC-MS

The samples for GC-MS analysis were analyzed for 2-(perfluorooctyl)ethanol under the following conditions. The concentration of 2-(perfluorooctyl)ethanol in the sample for GC-MS analysis was proportionally calculated by comparing the peak area on the mass fragmentogram of the sample for GC-MS analysis with that on the mass fragmentogram of 0.500 mg/L standard solution (see Table-6 and Fig.7).

The lowest detectable peak area was regarded as 300 considering the noise level, which corresponded to 0.021 mg/L of 2-(perfluorooctyl)ethanol.

(a) Analytical conditions

Instrument	Gas chromatograph–mass spectrometer Shimadzu Corporation type QP-5000

Conditions of GC

Column	HP-FFAP (Agilent) 25 m × 0.32 mm I.D., made of fused silica
Column temperature	50 °C (4 min)→170 °C (Rate 30 °C/min)
Carrier gas	Herium
Pressure of carrier gas	10.0 kPa
Injection temperature	200 °C
Sample size	2 μL
Sampling method	Splitless
Sampling time	2.0 min

Conditions of MS

Ionization method	Electron ionization (EI)
Detection mode	Positive ion
Monitoring method	Selected ion monitoring (SIM)
Monitoring m/z	95 (see Fig.9)
Interface temperature	230 °C

(b) Preparation of standard solution

The standard solution to determine the concentration of 2-(perfluorooctyl)-ethanol in the sample for GC-MS analysis was prepared as follows.

100 mg of the standard substance[*8] supplied by the sponsor was taken out accurately and dissolved in chloroform to obtain 2000 mg/L solution of 2-(perfluorooctyl)ethanol. 1.50 mg/L standard solution was then prepared from this solution by dilution with chloroform.

*8 Name, lot number and purity

Name	2-(perfluorooctyl)ethanol (A-1280)
Lot number	A18201X01
Purity	2-(perfluorooctyl)ethanol was treated as 100 % in purity.

(c) Calibration curve

0.375, 0.750 and 1.50 mg/L standard solutions were prepared by the same method as described in (b). These solutions were analyzed according to the analytical conditions described in (a). A calibration curve was drawn based on the relation between the peak area on the mass fragmentograms and the respective concentrations (see Fig.4).

4.5.3 Recovery test and blank test

Each two test solutions (water + 2-(perfluorooctyl)ethanol) and two test solutions (sludge + 2-(perfluorooctyl)ethanol) for recovery test were prepared according to the methods described in section 4.2. 0.300 mg of 2-(perfluorooctyl)ethanol was added to each test vessel, so that the concentration of it reached 1 mg/L. The test solutions were pretreated in accordance with the method described in section 4.5.1(2), then analyzed according to analytical conditions described in section 4.5.2(5).

A test solution for blank test was prepared according to the method described in section 4.2. The test solution for blank test was analyzed in the same way as the recovery test. As for the blank test, no peaks were appeared around the peak of the 2-(perfluorooctyl)ethanol on the mass fragmentogram.

Two individual recovery rates and their averages on the analytical procedure are shown below. The average recovery rates were used as correction factor, for the determination of 2-(perfluorooctyl)ethanol in the test solutions (see Table-5 and Fig.5).

Recovery rate of the test solutions (water + 2-(perfluorooctyl)ethanol)
99.6 %, 98.2 % average 98.9 %

Recovery rate of the test solutions (sludge + 2-(perfluorooctyl)ethanol)
80.5 %, 81.5 % average 81.0 %

Corrected page

4.6 Calculation of percentage biodegradation

The percentage biodegradation was calculated by the following equations and expressed in whole numbers.

(1) Percentage biodegradation by BOD

$$\text{Percentage biodegradation (\%)} = \frac{BOD - B}{TOD^{*9}} \times 100$$

BOD	:	Biochemical oxygen demand in the test solution (sludge + test item) (experimental) (mg)
B	:	Biochemical oxygen demand in the control blank (experimental) (mg)
TOD^{*9}	:	Theoretical oxygen demand required when the test item was completely oxidized (theoretical) (mg)

*9 The purity was regarded as 100 % and TOD was calculated from composition formula (CBI) which was calculated by use of n=8, monomer values of (CBI) regarded as mol% in section 1.2.

(2) Percentage biodegradation by weight measurement

$$\text{Percentage biodegradation (\%)} = \frac{Sw - Ss}{Sw} \times 100$$

Ss	:	Residual amount of the test item in the test solution (sludge + test item) (experimental) (mg)
Sw	:	Residual amount of the test item in the test solution (water + test item) (experimental) (mg)

4.7 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401:1999 rule B.

5 Validity of test conditions

Percentage biodegradations of aniline calculated by the BOD values were 68 % and 72 % after 7 and 14 days, respectively It was concluded that this test conditions were valid (see Table-1 and Fig 1).

6. Factors possibly affecting accuracy

No adverse effects on the reliability of this test were noted.

7 Results

7.1 Appearances of test solutions

Appearances of test media in cultivation vessels were as follows.

	Test solution	Appearance	pH
At the start of cultivation	Water + test item	The test item was not dissolved.	-
	Sludge + test item	The test item was not dissolved.	-
At the termination of cultivation	Water + test item	Insoluble compound was observed.	-
	Sludge + test item	Insoluble compound except for the sludge was observed. Growth of the sludge was not observed.	-

7.2 Analytic results of test solutions

Analytic results of the test solution after 28 days were as follows.

		Water + test item	Sludge + test item			Theoretical amount	Table	Fig.
		Vessel-6	Vessel-1	Vessel-2	Vessel-3			
BOD*10	mg	0.1	0.6	1.1	1.8	(CBI)	1	1
*10 Detection amount and percentage detection of DOC	mgC	0	0	0.3	0.3	(CBI)	2	-
	%	0	0	3	3	-		
Residual amount and percentage residue of test item (Weight measurement)	mg	28.5	30.0	29.6	28.9	30.0	3	-
	%	95	100	99	96	-		
*11 Concentration of perfluorooctanoic acid (LC-MS)	mg/L	≦0.020	≦0.020	≦0.020	≦0.020	-	4	6

		Water + test item	Sludge + test item			Theoretical amount	Table	Fig.
			1	2	3			
*12 Concentration of 2-(perfluorooctyl) ethanol (GC-MS)	mg/L	≦0.014	≦0.017	≦0.017	≦0.017	-	6	7

*10 The value of control blank was subtracted from the values of the test solutions (sludge + test item)

*11 Minimum determination limit of perfluorooctanoic acid in test solution
= (Minimum determination limit in sample for LC-MS analysis) ×
{(final volume) / (volume of test solution)} / (ratio of portion used for analysis)
= 0.0098 mg/L × (5 mL/300 mL) / (2.5/300) = 0.020 mg/L

*12 Minimum determination limit of 2-(perfluorooctyl)ethanol in test solution
= (Minimum determination limit in sample for GC-MS analysis) ×
{(final volume) / (volume of test solution)} / {(average recovery rate) / 100} /
(ratio of portion used for analysis)
Test solution (water + test item) :
0.021 mg/L × (200 mL / 300 mL) / (98.9/100) / 1 = 0.014 mg/L
Test solution (sludge + test item) :
0.021 mg/L × (200 mL / 300 mL) / (81.0/100) / 1 = 0.017 mg/L

7.3 Percentage biodegradation

Percentage biodegradations after 28 days were as follows

Method	Percentage biodegradation (%)				Table
	Vessel-1	Vessel-2	Vessel-3	Average	
BOD	2	4	6	4	1
Weight measurement	0	0	0	0	3

7.4 Results of measurement of IR spectrum

No significant difference between IR spectrum of the test item and that of the residue after weight measurement was observed (see Figs.2, 10).

7.5 Discussion

Determination of the test item by chromatography was difficult, because the test item was not completely dissolved in water and organic solvents (tetrahydrofuran and chroloform). Therefore, the test item was determined by means of weight measurement. As a result, the percentage residues of the test item were 95 % in the test solution (water + test item) and 100, 99 and 96 % in the test solutions (sludge + test item). The percentage detections of DOC were 0 % in the test solution (water + test item) and 0, 3 and 3 % in the test solutions (sludge + test item). These results indicate that the test item was not dissolved in the test solution.

No significant difference between IR spectra of the test item before and after the cultivation was observed. Converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected to form in test solutions, was not detected. These results indicate that the test item was not converted during the cultivation.

It is considered that the test item was not biodegraded by microorganisms, because the average percentage biodegradation by BOD was 4 % and no significant change in the weight of the test item before and after the cultivation was observed.

7.6 Conclusion

The test item was not biodegraded by microorganisms under the present test conditions.

8. Remarks

8.1 Instruments used for test

Fourier transform infrared spectrophotometer :
Shimadzu Corporation type FTIR-8200PC

Closed system oxygen consumption measuring apparatus :
see page 11

Total organic carbon analyzer : see page 14

Liquid chromatograph- mass spectrometer :
see page 15

Gass chromatograph- mass spectrometer :
see page 17

Electronic analytical balance : Mettler Toledo type AT201
Sartorius AG type CP324S
Sartorius AG type BP210S

Refrigerated centrifuge : Shimadzu Corporation type CST-060LF

Mechanical shaker : TIETECH Co., Ltd. type SR-2w

Vaccume oven : SIBATA SCIENTIFIC TECHNOLOGY LTD. type VS-300

8.2 Reagents used for analysis

Methanol (HPLC grade) : Wako Pure Chemical Industries, Ltd.

Purified water : Takasugi Seiyaku Co., Ltd.

Chloroform (reagent grade) : Kishida Chemical Co., Ltd.

Ammonium sulfate (reagent grade): Kanto Chemical Co., Inc.

Sodium sulfate (reagent grade): Kanto Chemical Co., Inc.

Formic acid (reagent grade): Wako Pure Chemical Industries, Ltd.

Sodium hydrogencarbonate (reagent grade):
Wako Pure Chemical Industries, Ltd.

Sodium carbonate (reagent grade): Wako Pure Chemical Industries, Ltd.

Potassium hydrogenphthalate (reagent grade) :
Wako Pure Chemical Industries, Ltd.

Potassium bromide (for measurement of infrared absorption) :
NACALAI TESQUE, INC.

Perfluorooctanoic acid : Standard substance supplied by the sponsor

2-(perfluorooctyl)ethanol : Standard substance supplied by the sponsor

Table 1
Calculation table for the percentage biodegradation by BOD.

Test No. 14114 . Cultivating Duration: 28 days

Vessel No.	7th day		14th day		21st day		28th day		Mean Deg. %
	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	
④	63.6	68	67.8	72	69.6	73	71.8	75	
⑤	2.0	-	2.6	-	3.4	-	4.5	-	
①	2.7	2	3.3	2	4.0	2	5.1	2	4
②	2.1	0	2.7	0	3.7	1	5.6	4	
③	3.0	3	3.6	3	4.3	3	6.3	6	
⑥	0.0	-	0.0	-	0.0	-	0.1	-	

Deg. : Percentage biodegradation

Vessel no. ④ : Sludge + Aniline
Vessel no. ⑤ : Control blank [B]
Vessel no. ① ② ③ : Sludge + Test substance
Vessel no. ⑥ : Water + Test substance

Test substance of 30 mg was added.

Chart of BOD : Fig. 1

Deg. = [BOD - B] / [TOD] × 100 (%)

TOD of test substance : (CBI) (mg)

TOD = 30.0 × (CBI) = (CBI) (mg)

TOD of aniline : 90.3 (mg)

$C_6H_7N + 8.75\ O_2 \rightarrow 6\ CO_2 + 3.5\ H_2O + NO_2$

$8.75\ O_2 / C_6H_7N = 279.99 / 93.13 = 3.01$

TOD = 30.0 × 3.01 = 90.3 (mg)

2003.08.15 Name K. Ogawa

Table-2 Calculation table for amount of dissolved organic carbon by TOC

Study No. 14114

Sample description	A	B	C	D
Water blank	n.d.			
[6] Water + test item	n.d.	0	0	0
[1] Sludge + test item	n.d.	0	0	
[2] Sludge + test item	1.15	0.3	3	2
[3] Sludge + test item	1.02	0.3	3	
[5] Control blank	n.d.			

Amount of test item added : 30 (mg)

Volume of test solution : 300 (mL)

Theoretical amount of carbon :(CBI) (mgC) (30) × (CBI)

A : Measured value (mgC/L)

B : Amount of DOC

Bw = (A(Water + test item) - A(Water blank)) × 300 / 1000 (mgC)

Bs = (A(Sludge + test item) - A(Control blank)) × 300 / 1000 (mgC)

C : Percentage detection (%)

C = B / (Theoretical amount of carbon) × 100 (%)

D : Average percentage detection (%)

August 26, 2003 Name K. Ogawa

Table-3 Calculation table for percentage biodegradation by weight measurement

Study No. 14114

Sample description	A	B	C	D	E
[6] Water + test item	28.5	28.5	95		
[1] Sludge + test item	33.9	30.0	100	0	
[2] Sludge + test item	33.5	29.6	99	0	0
[3] Sludge + test item	32.8	28.9	96	0	
[5] Control blank	3.9				

Amount of test item added : 30 (mg)

Volume of test solution : 300 (mL)

A : Weight of residue (mg)

B : Residual amount of test item (mg)

Bw = A(Water + test item)

Bs = A(Sludge + test item) - A(Control blank)

C : Percentage residue (%)

C = B / (Amount of test item added) × 100

D : Percentage biodegradation (%)

D = (Bw - Bs) / Bw × 100

E : Average percentage biodegradation (%)

August 26, 2003 Name K. Oyama

Table-4 Calculation table for concentration of perfluorooctanoic acid by LC-MS

Study No. 14114

Sample description	A	D	E
Standard solution 0.500mg/L	102018		
[6] Water + test item	n.d.	0 (≦0.020)	-
[1] Sludge + test item	n.d.	0 (≦0.020)	
[2] Sludge + test item	n.d.	0 (≦0.020)	-
[3] Sludge + test item	n.d.	0 (≦0.020)	
[5] Control blank	n.d.		

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 5 (mL)

C : Ratio of portion used for analysis : 2.5/300

D : Concentration of perfluorooctanoic acid in test water (mg/L)

$D = F \times (A / A(Standard)) \times (B / 300) / C$

Minimum determination limit : 0.020 (mg/L)

E : Average concentration of perfluorooctanoic acid (mg/L)

F : Concentration of standard solution : 0.500 (mg/L)

See Fig. 6

September 18, 2003 Name K. Oyama

Table-5 Calculation table for recovery rate by GC-MS
(2-(perfluorooctyl)ethanol)

Study No. 14114

Sample description	A	D	E	F
Standard solution 1.50mg/L	26329			
Water + 2-(perfluorooctyl)ethanol -1	26222	0.299	99.6	98.9
Water + 2-(perfluorooctyl)ethanol -2	25861	0.295	98.2	
Sludge + 2-(perfluorooctyl)ethanol -1	21196	0.242	80.5	81.0
Sludge + 2-(perfluorooctyl)ethanol -2	21459	0.245	81.5	
Control blank	n.d.			

Amount of 2-(perfluorooctyl)ethanol added : 0.300 (mg)

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 200 (mL)

C : Ratio of portion used for analysis : 1

D : Recovery amount (mg)

$D = G \times (A / A(\text{Standard})) \times (B / 300) / C$

E : Recovery rate (%)

$E = D / 0.300 (\text{mg}) \times 100$

F : Average recovery rate (%)

See Fig. 5

September 11, 2003 Name k. Iyama

Table-6 Calculation table for concentration of 2-(perfluorooctyl)ethanol by GC-MS

Study No. 14114

Sample description	A	E	F
Standard solution 1.50mg/L	20965		
Water + test item	n.d.	0 (≦0.014)	-
Sludge + test item -1	n.d.	0 (≦0.017)	
Sludge + test item -2	n.d.	0 (≦0.017)	-
Sludge + test item -3	n.d.	0 (≦0.017)	
Control blank	n.d.		

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 200 (mL)

C : Ratio of portion used for analysis : 1

D : Recovery rate : 98.9 (%) (Water + test item)
81.0 (%) (Sludge + test item)

E : Concentration of 2-(perfluorooctyl)ethanol in test solution (mg/L)

$E = G \times (A / A(Standard)) \times (B / 300) / (D / 100) / C$

Minimum determination limit : 0.014 (mg/L) (Water + test item)
0.017 (mg/L) (Sludge + test item)

F : Average concentration of 2-(perfluorooctyl)ethanol (mg/L)

G : Concentration of standard solution : 1.50 (mg/L)

See Fig. 7

September 18, 2003 Name K. Oyama

Fig.1 Chart of BOD

Test No. 14114 (Test substance CBI)

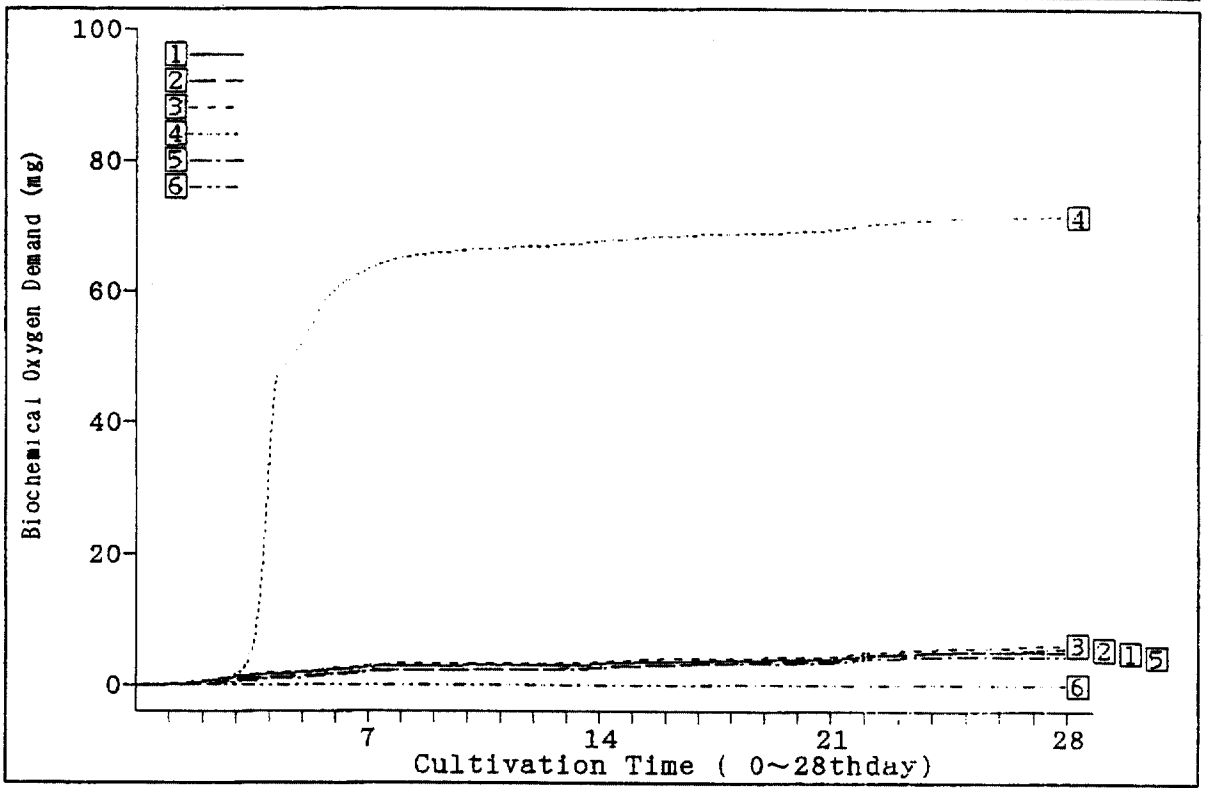
Apparatus No. CM-17

Cultivating conditions:

Concentration	
Test substance	100 (mg/ℓ)
Reference substance(aniline)	100 (mg/ℓ)
Activated sludge	30 (mg/ℓ)
Temperature	25 ± 1°C
Duration	28days(Jul.18~Aug.15,2003)

Note: Regular condition

Vessel no.	Sample description	B O D (mg)			
		7thday	14thday	21stday	28thday
1	Sludge + Test substance	2.7	3.3	4.0	5.1
2	Sludge + Test substance	2.1	2.7	3.7	5.6
3	Sludge + Test substance	3.0	3.6	4.3	6.3
4	Sludge + Aniline	63.6	67.8	69.6	71.8
5	Control blank [B]	2.0	2.6	3.4	4.5
6	Water + Test substance	0.0	0.0	0.0	0.1



2003.08.15 Name K. Ogawa

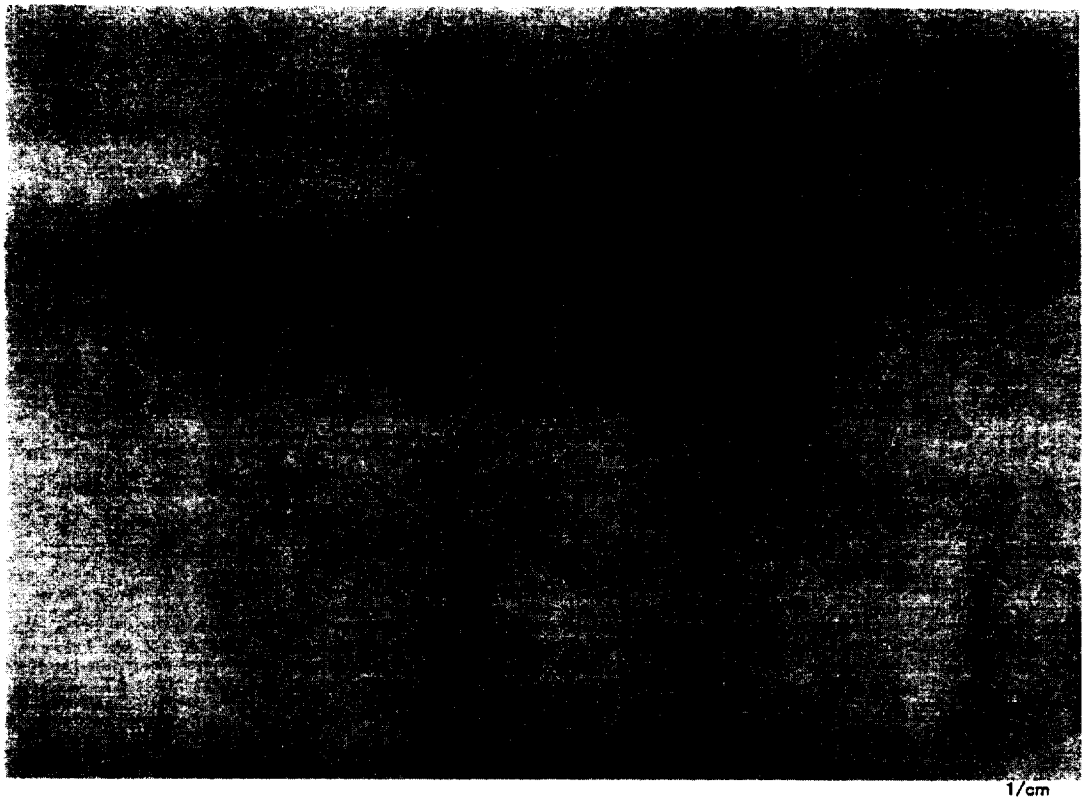


Fig. 2-1 IR spectrum of residue.

Study No. : 14114
Sample : [6] Water + test item
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

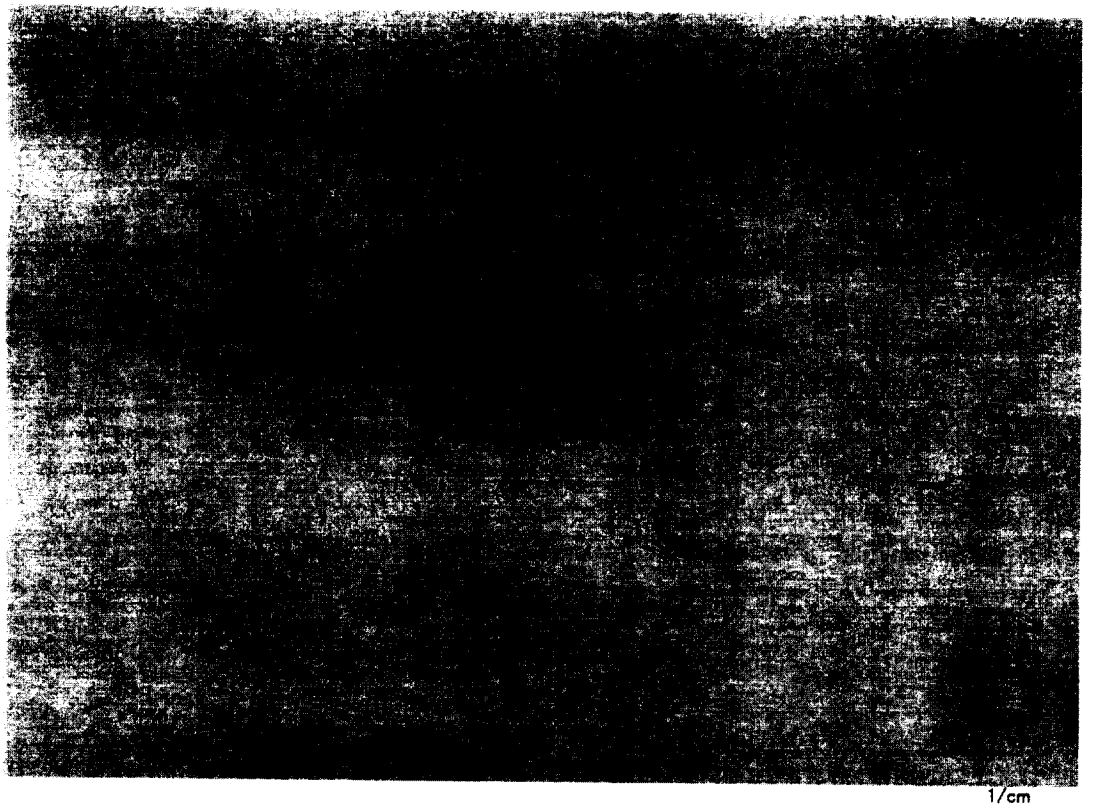


Fig.2 -2 IR spectrum of residue.

Study No. : 14114
Sample : [1] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : K. Oyama

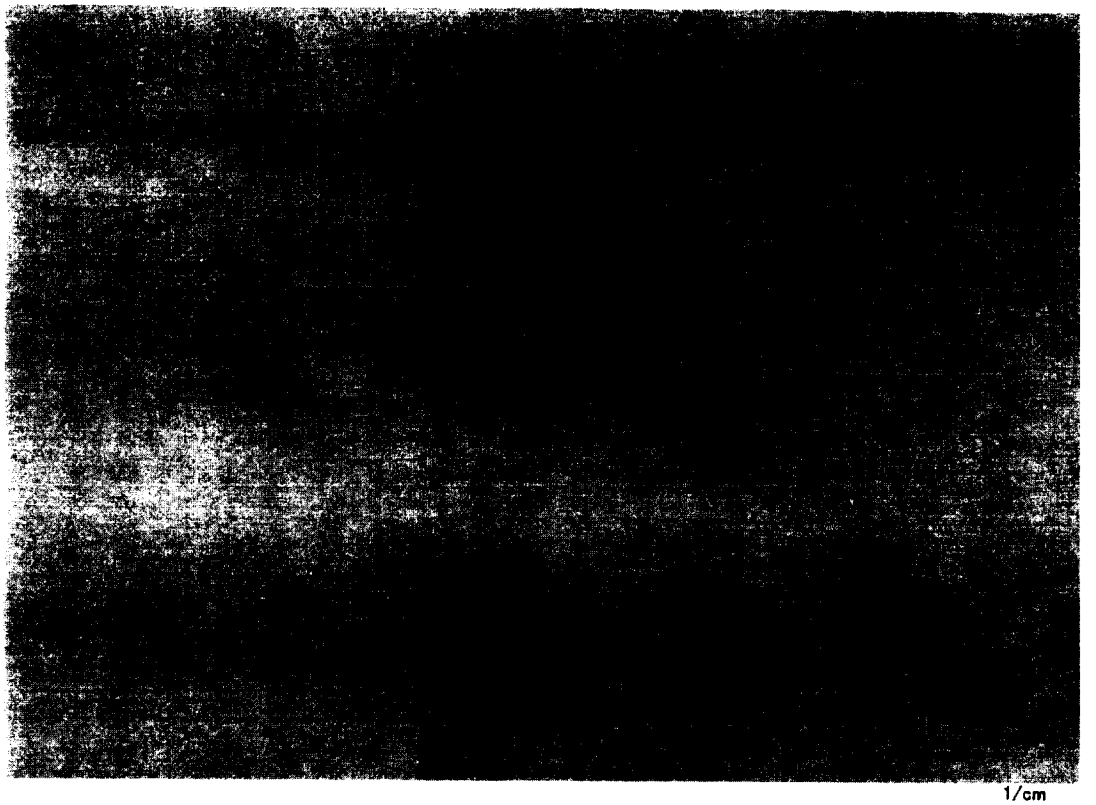


Fig.2 -3 IR spectrum of residue.

Study No. : 14114
Sample : [2] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

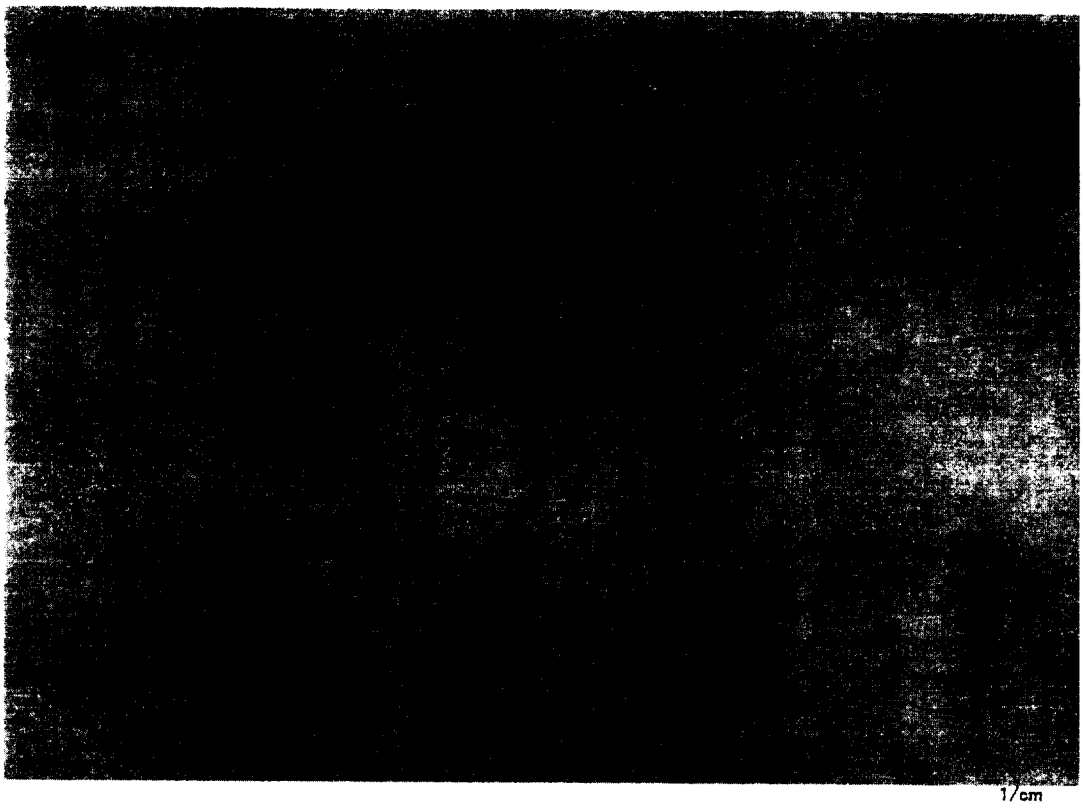


Fig. 2 -4 IR spectrum of residue.

Study No. : 14114
Sample : [3] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

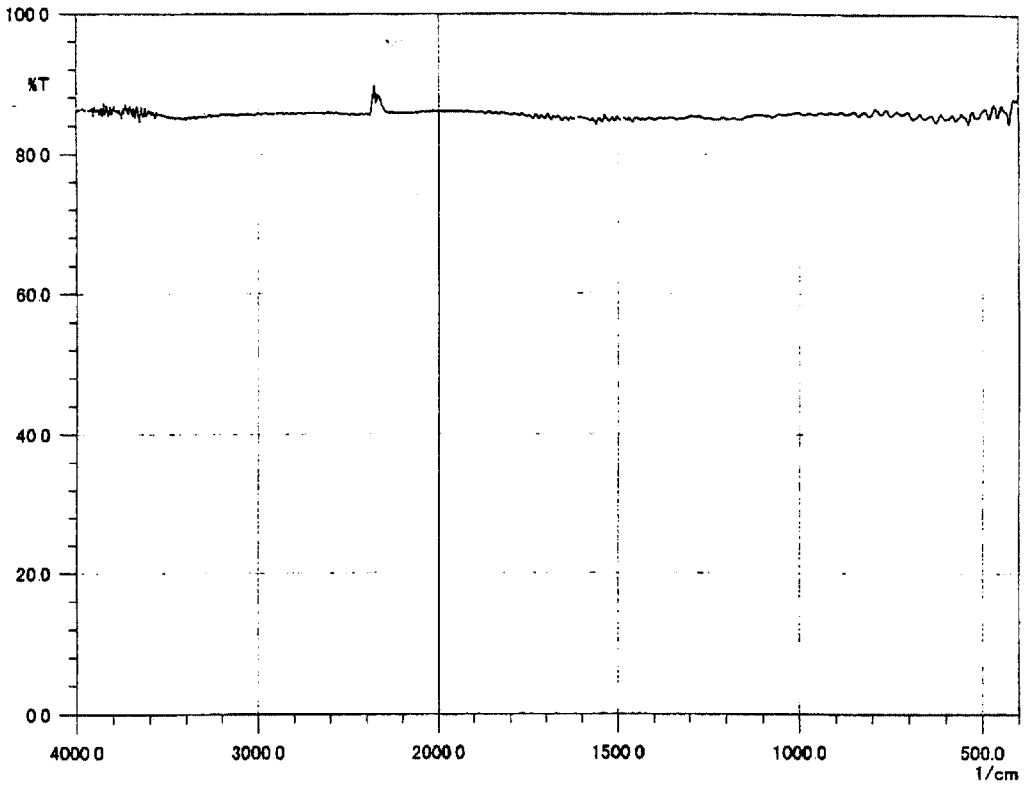


Fig. 2 -5 IR spectrum of residue.

Study No. : 14114
Sample : [5] Control blank
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

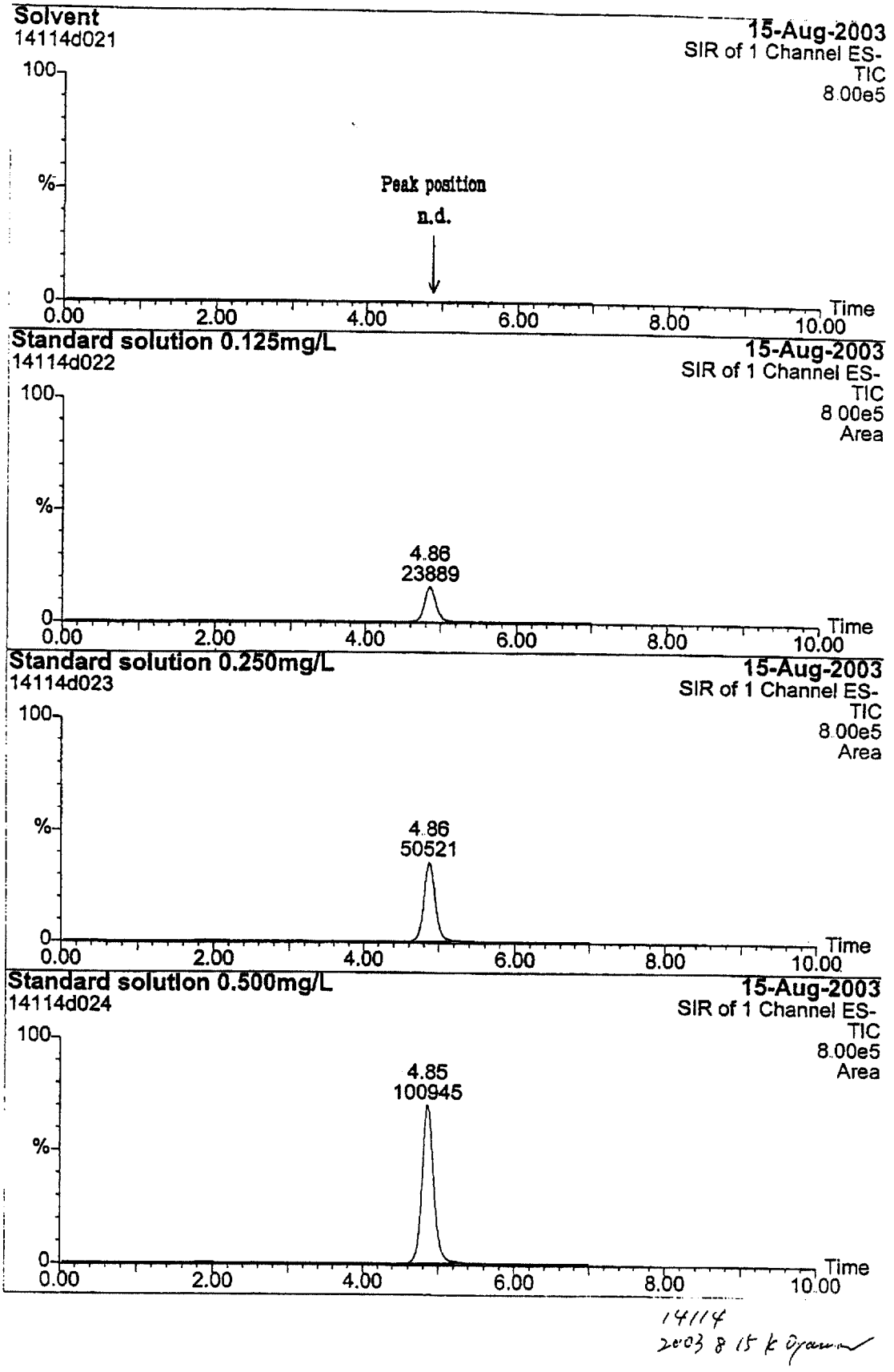
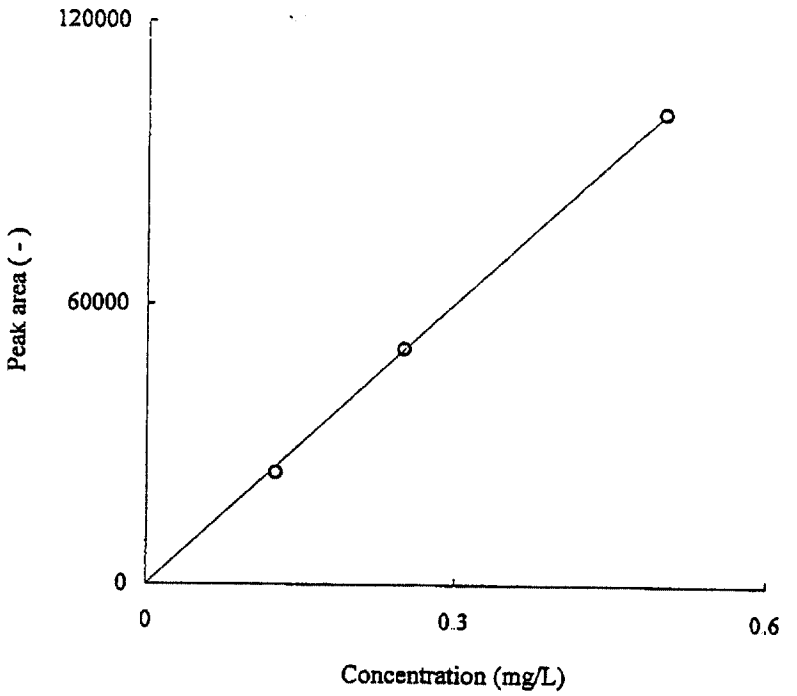


Fig.3 - 1 Mass fragmentogram of LC-MS analysis for calibration curve (perfluorooctanoic acid).



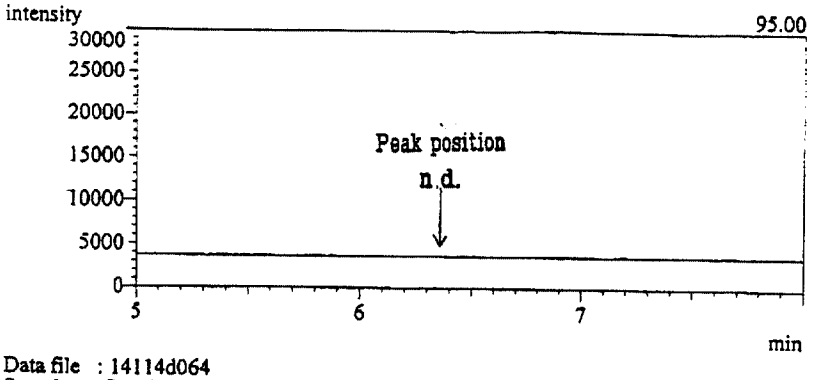
y = 201414x
r = 1.00

Concentration (mg/L)	Peak area (-)
0.125	23889
0.250	50521
0.500	100945

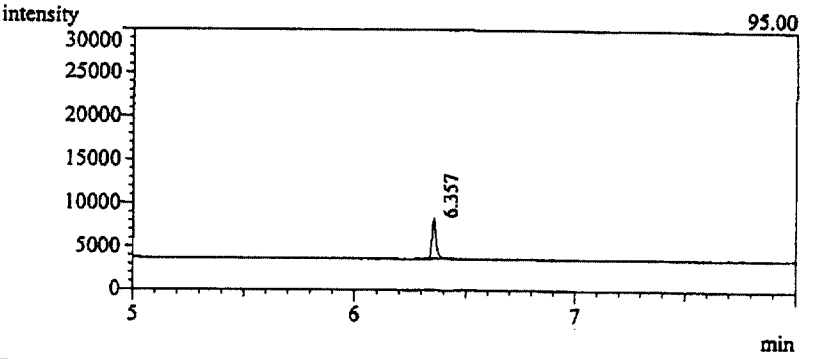
Fig. 3 - 2 Calibration curve of LC-MS analysis for perfluorooctanoic acid

August 22, 2003 Name K. Ojama

Data file : 14114d063
Sample : Solvent

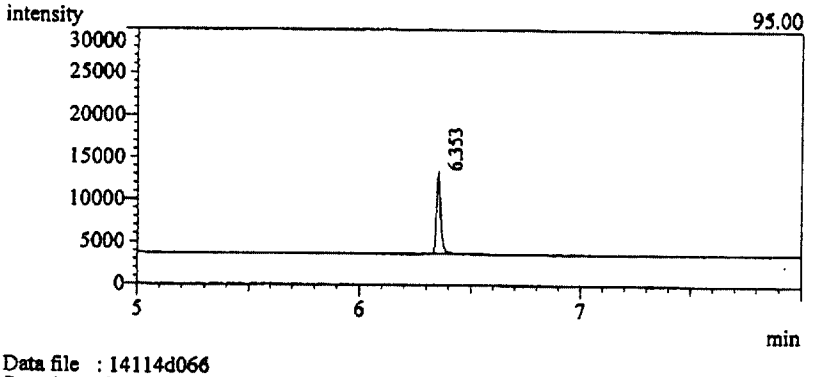


Data file : 14114d064
Sample : Standard solution 0.375mg/L



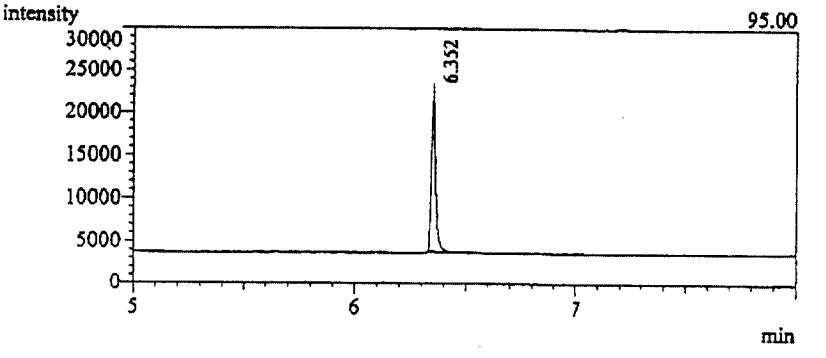
Peak	R.T.	Peak area
1	6.357	6136

Data file : 14114d065
Sample : Standard solution 0.750mg/L



Peak	R.T.	Peak area
1	6.353	12339

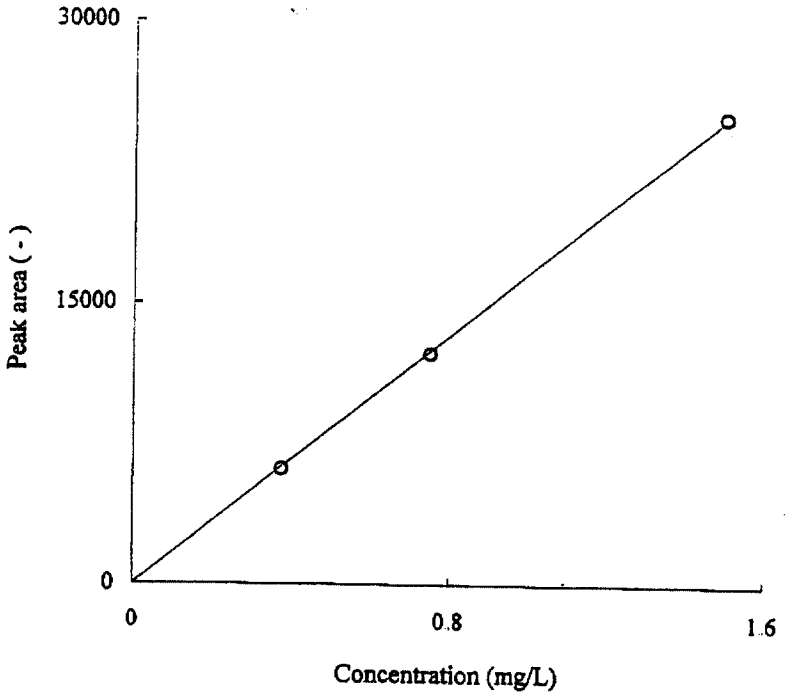
Data file : 14114d066
Sample : Standard solution 1.50mg/L



Peak	R.T.	Peak area
1	6.352	25069

Date : August 7, 2003 Name : K. Ogama

Fig.4 - 1 Mass fragmentogram of GC-MS analysis for calibration curve (2-(perfluorooctyl)ethanol)



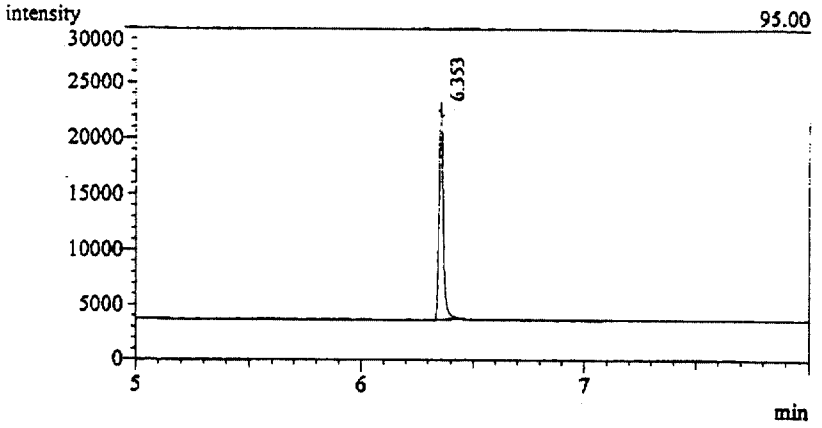
y = 16646x
r = 1.00

Concentration (mg/L)	Peak area (-)
0.375	6136
0.750	12339
1.50	25069

Fig. 4 - 2 Calibration curve of GC-MS analysis for 2-(perfluorooctyl)ethanol

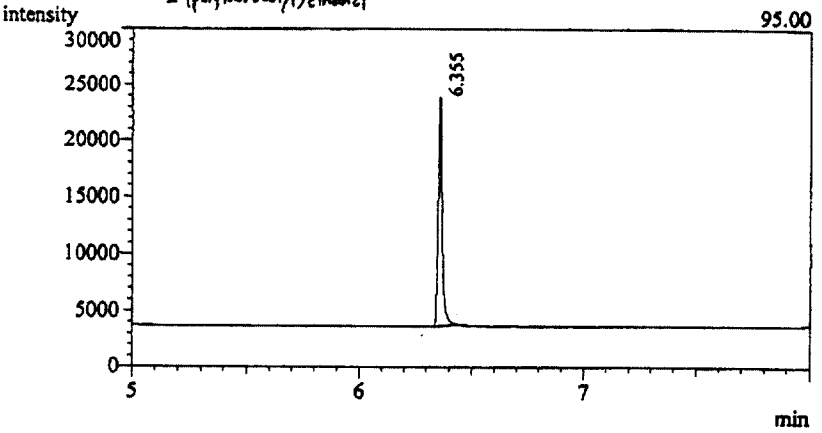
August 11, 2003 Name K. Oyama

Data file : 14114d067
Sample : Standard solution 1.50mg/L



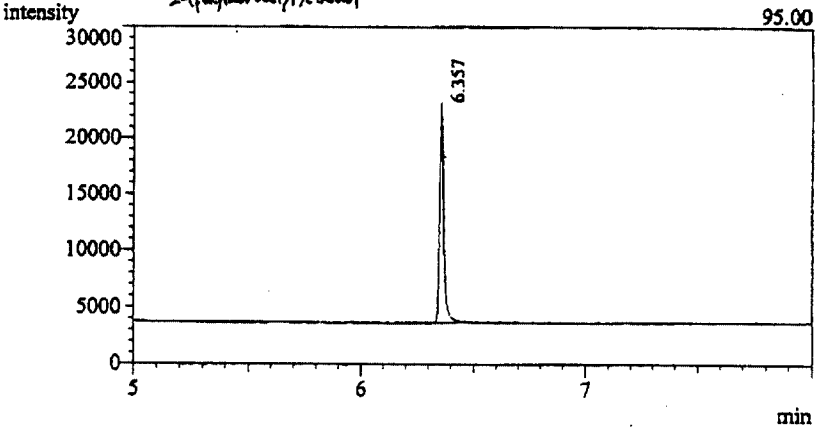
Peak	R.T.	Peak area
1	6.353	26329

Data file : 14114d068
Sample : Water + ~~test item~~ -1
2-(perfluorooctyl)ethanol



Peak	R.T.	Peak area
1	6.355	26222

Data file : 14114d069
Sample : Water + ~~test item~~ -2
2-(perfluorooctyl)ethanol

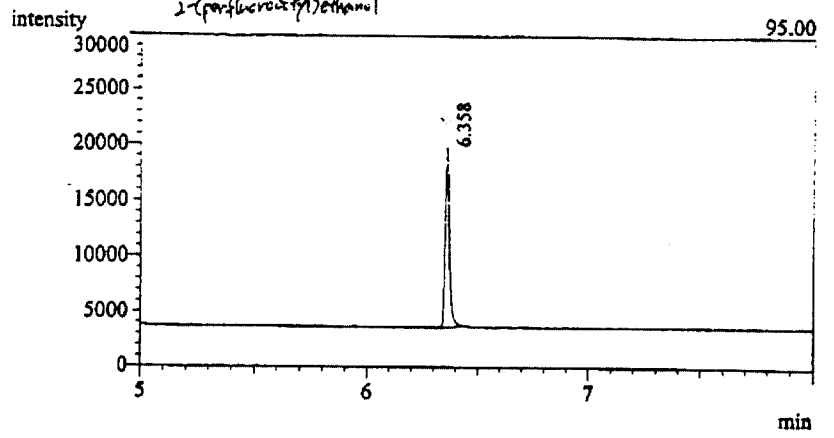


Peak	R.T.	Peak area
1	6.357	25861

Date : August 7, 2003 Name : K. Ogawa

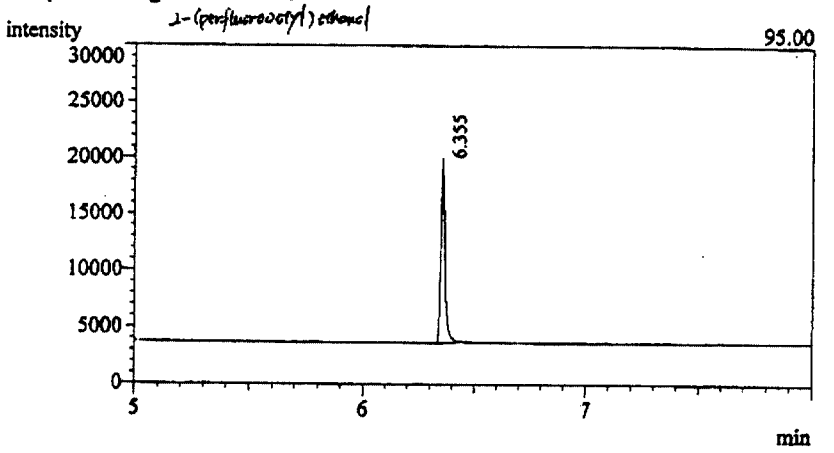
Fig.5 - 1 Mass fragmentogram of GC-MS analysis for recovery and blank test (analysis of 2-(perfluorooctyl)ethanol).

Data file : 14114d070
Sample : Sludge + ~~test item~~ -1



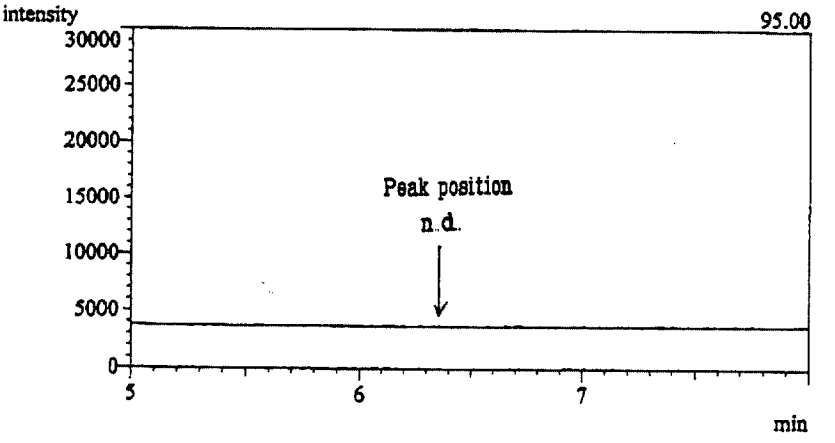
Peak	R.T.	Peak area
1	6.358	21196

Data file : 14114d071
Sample : Sludge + ~~test item~~ -2



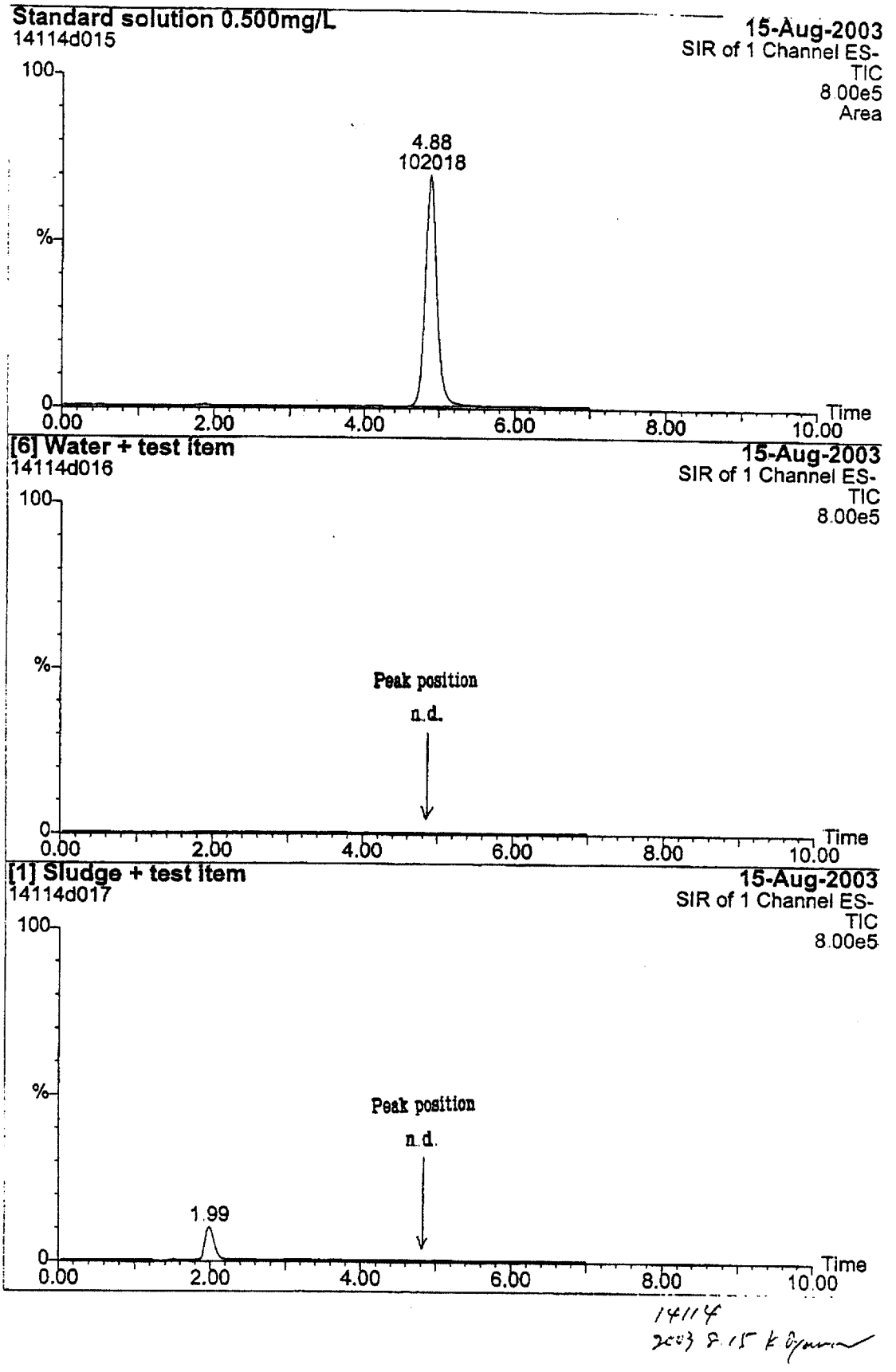
Peak	R.T.	Peak area
1	6.355	21459

Data file : 14114d072
Sample : Control blank



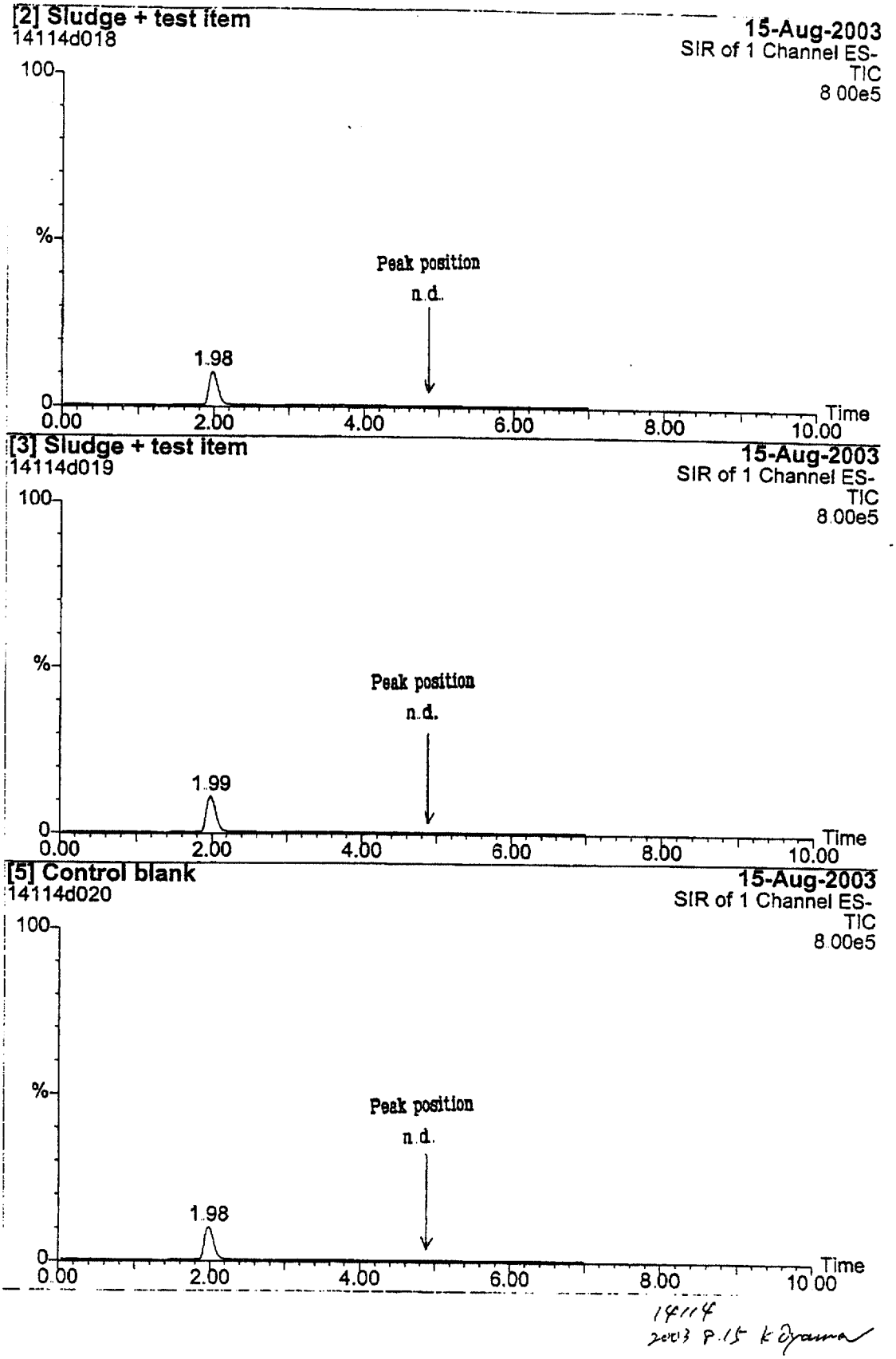
Date : August 7, 2003 Name : K. Oyama

Fig.5 - 2 Mass fragmentogram of GC-MS analysis for recovery and blank test (analysis of 2-(perfluorooctyl)ethanol).



14114
2003 8.15 k Oyama

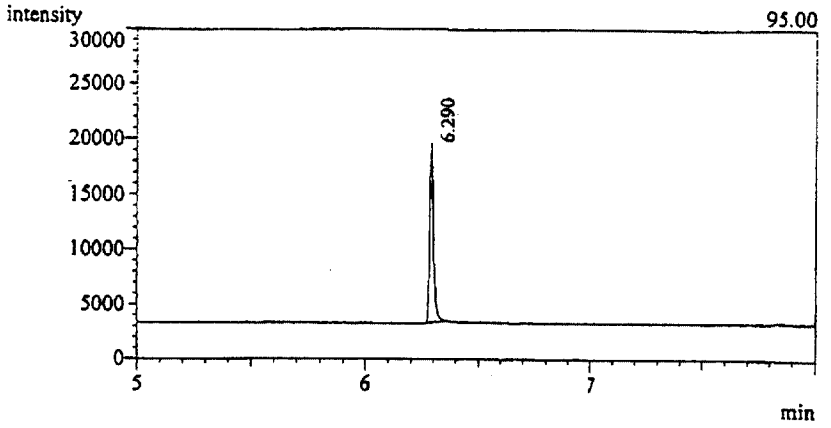
Fig.6 - 1 Mass fragmentogram of LC-MS analysis for test solution (perfluorooctanoic acid).



14114
2003 P.15 K Ojima

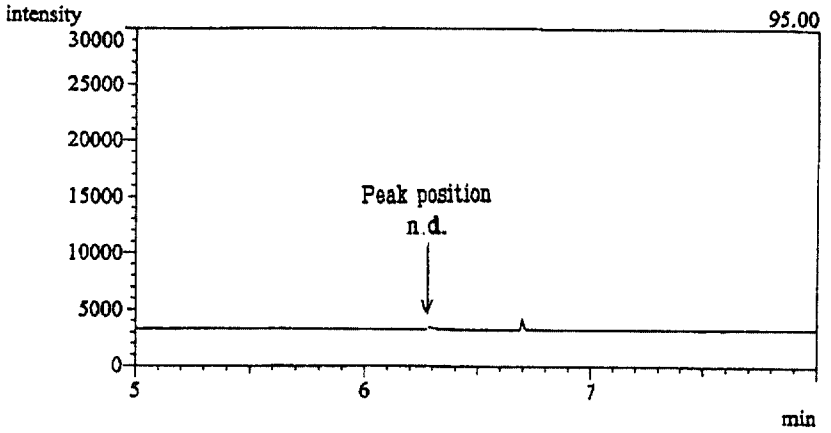
Fig.6 - 2 Mass fragmentogram of LC-MS analysis for test solution (perfluorooctanoic acid).

Data file : 14114d074
Sample : Standard solution 1.50mg/L

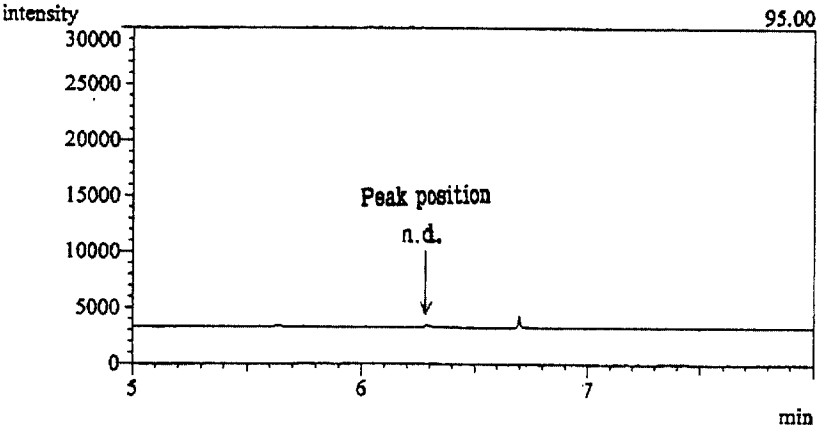


Peak	R.T.	Peak area
1	6.290	20965

Data file : 14114d075
Sample : Water + test item



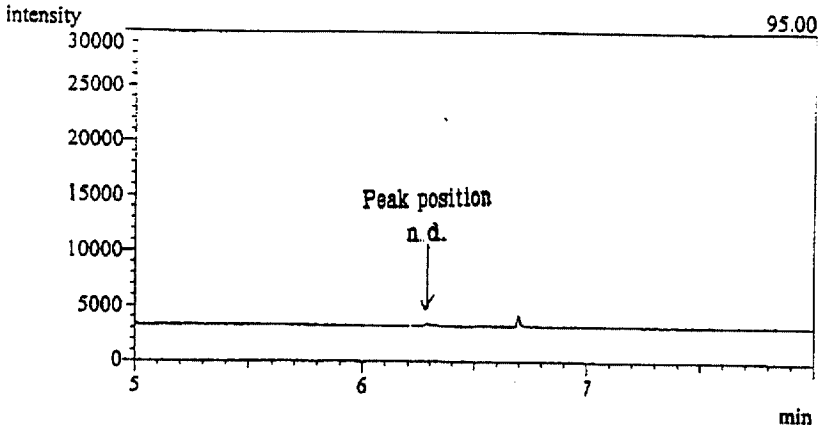
Data file : 14114d076
Sample : Sludge + test item -1



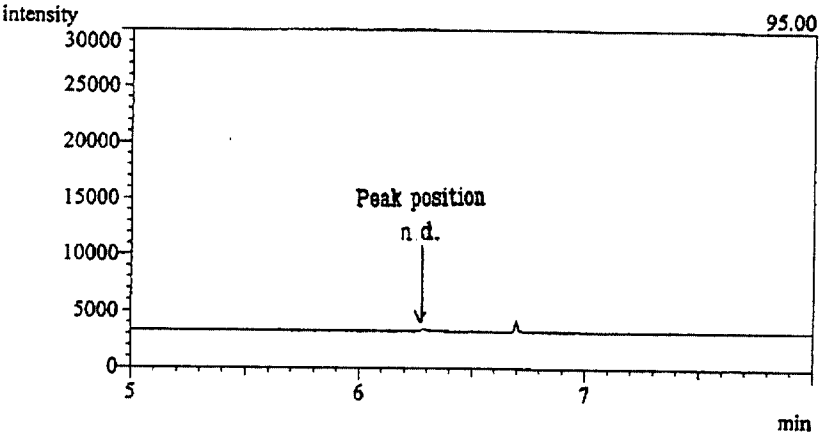
Date : August 18, 2003 Name : K. Ogawa

Fig.7 - 1 Mass fragmentogram of GC-MS analysis for test solution (2-(perfluorooctyl)ethanol).

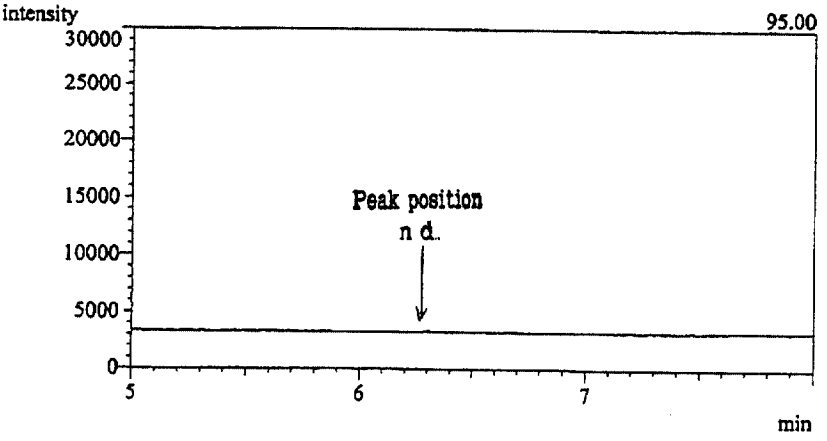
Data file : 14114d077
Sample : Sludge + test item -2



Data file : 14114d078
Sample : Sludge + test item -3



Data file : 14114d079
Sample : Control blank



Date : August 18, 2003 Name : K. Ojama

Fig.7 - 2 Mass fragmentogram of GC-MS analysis for test solution (2-(perfluorooctyl)ethanol).

Study No. 14114

Instrument MS : Micromass Quattro II, HPLC : Jasco PU-980, AS-950

Sample Perfluorooctanoic acid

HPLC Conditions

Column L-column ODS

Size 15 cm x 4.6 mm I.D.

Eluent Methanol*1/purified water*1 (8/2 V/V) *1 containing 0.1% formic acid

Flow rate 1.0 mL/min

Sample size 20 μL (Solvent Methanol)

MS Conditions

Ionization mode ESI

Detection mode Negative

Function MS

Source conditions (Capillary 3.0 kV, Cone 20 V, Temperature 150 °C)

Mass range (m/z) 100 – 1000

Date June 17, 2003 Operator K. Oyama

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan

Fig.8 - 1 Mass spectrum of perfluorooctanoic acid (analytical condition).

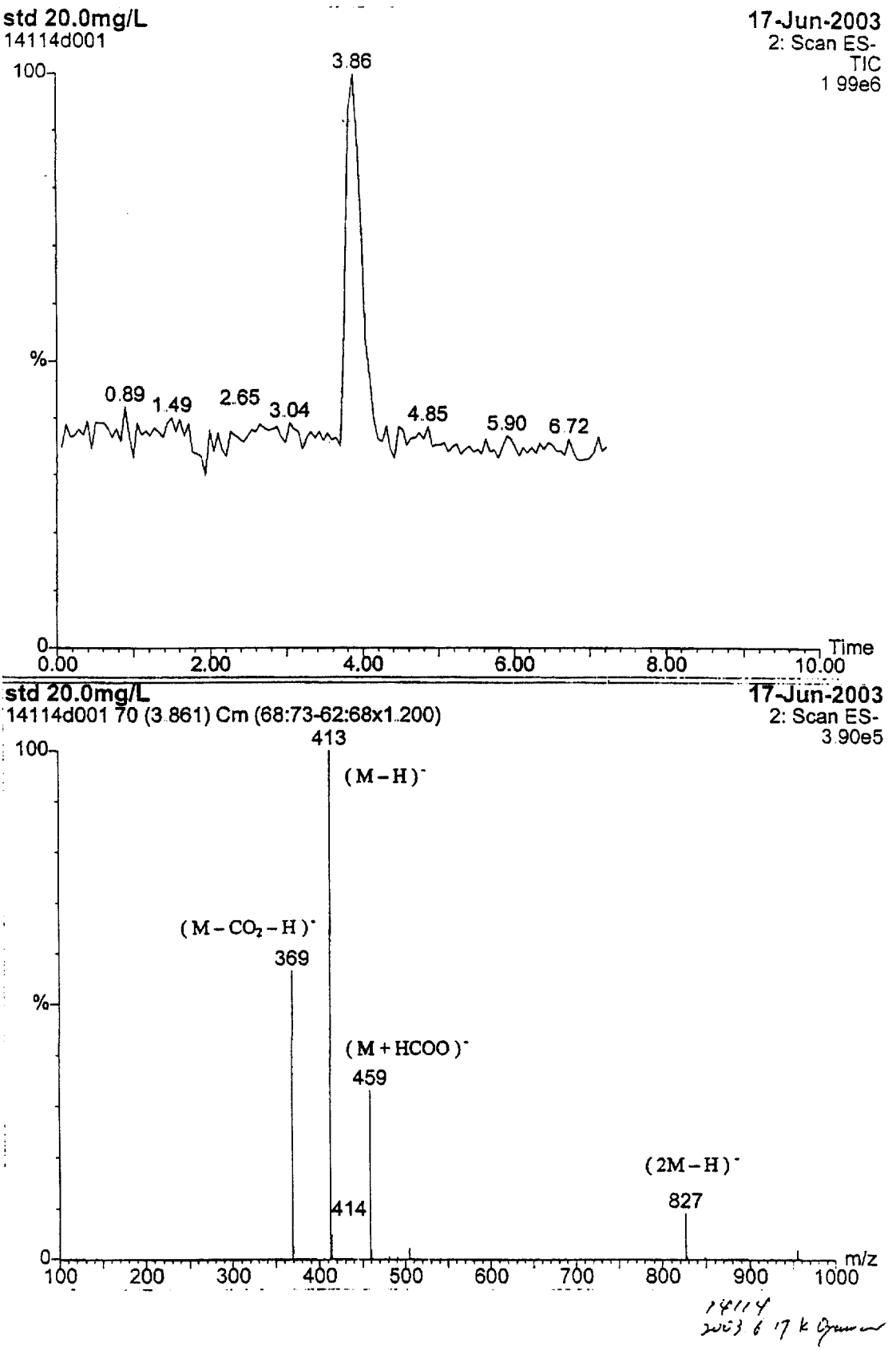


Fig.8 - 2 Mass spectrum of perfluorooctanoic acid (mass spectrum).

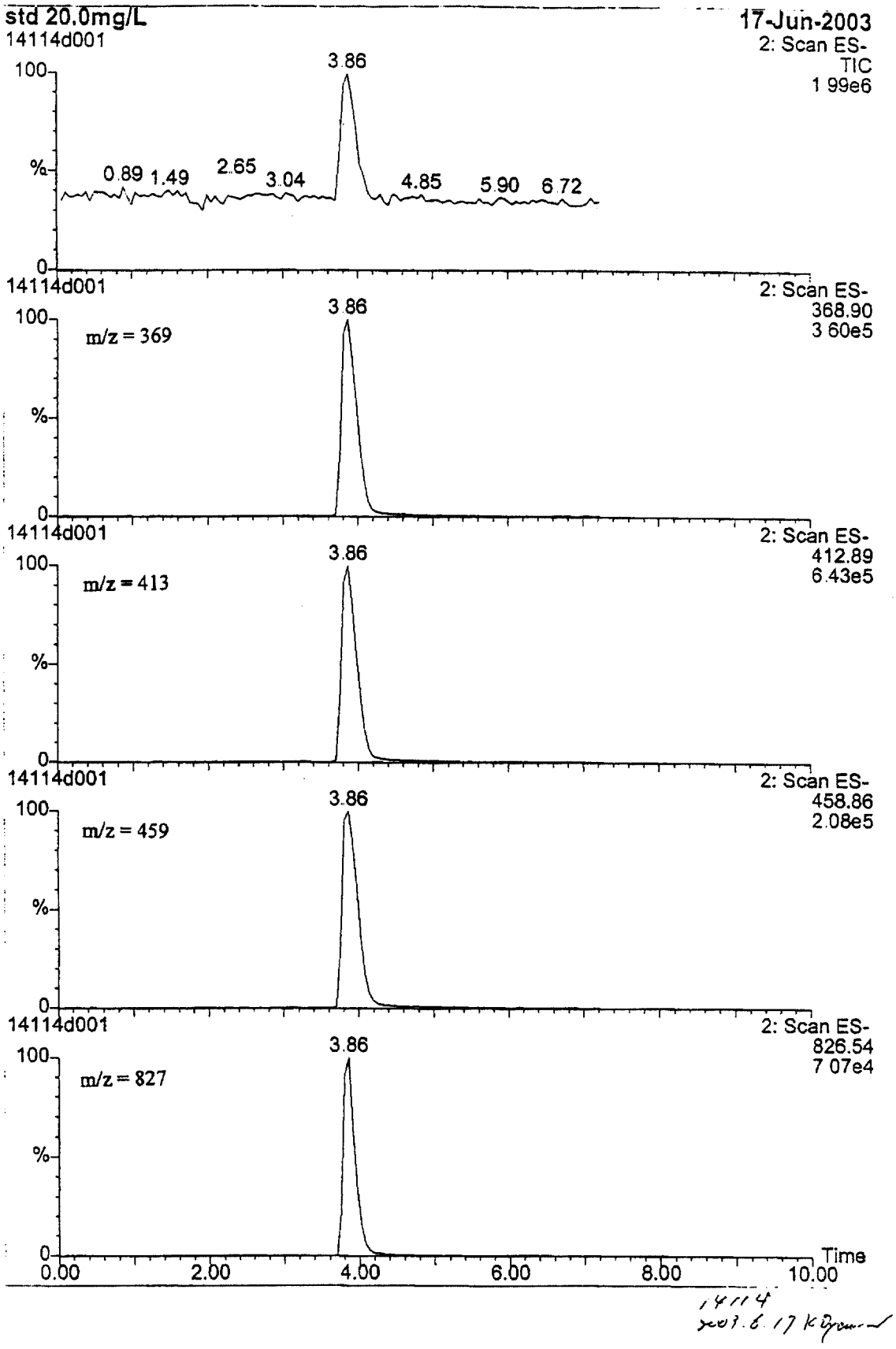


Fig.8 - 3 Mass spectrum of perfluorooctanoic acid (mass chromatogram)

Study No. 14114

Instrument Shimadzu QP-5000

Sample 2-(perfluorooctyl)ethanol

GC Conditions

Column HP-FFAP (Fused silica)

Size 25 m x 0.32 mm I.D., Film thickness 0.52 μm

Column temp. 50 °C (3 min) → 170 °C, Temp. rate 30 °C/min

Carrier gas He

Column head pressure 10.0 kPa

Injection temp. 200 °C

Sample size 1 μL (Solvent Methanol)

Inlet mode Splitless

Sampling time 2.0 min

MS Conditions

Ionization Mode EI

Detection Mode Positive

Mass range (m/z) 50 - 600

Interface temp. 230 °C

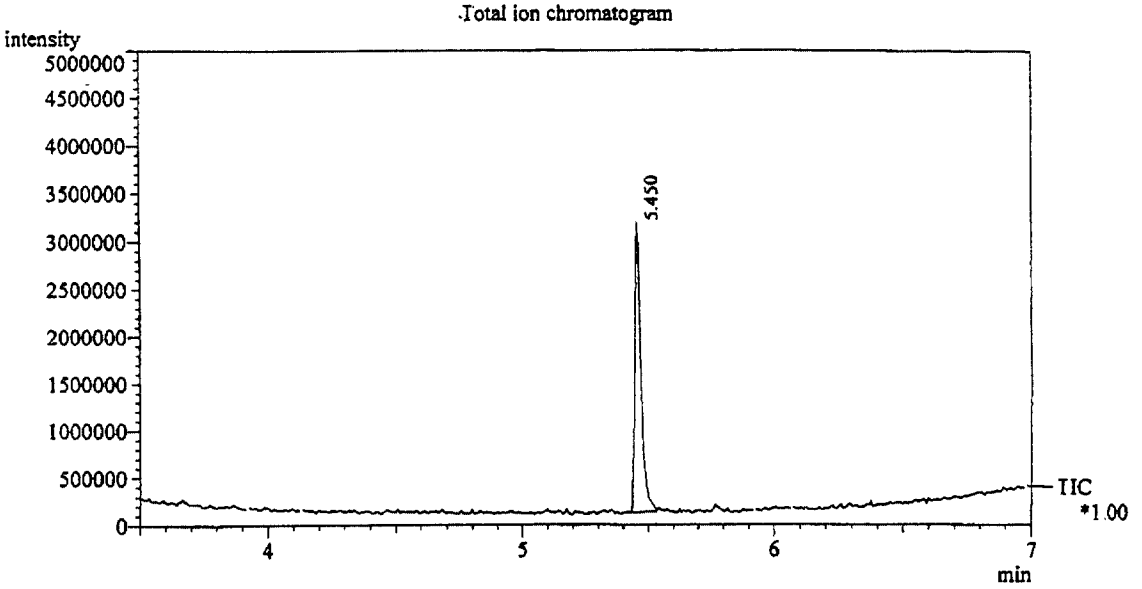
Date June 11, 2003 Operator K. Ogawa

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan

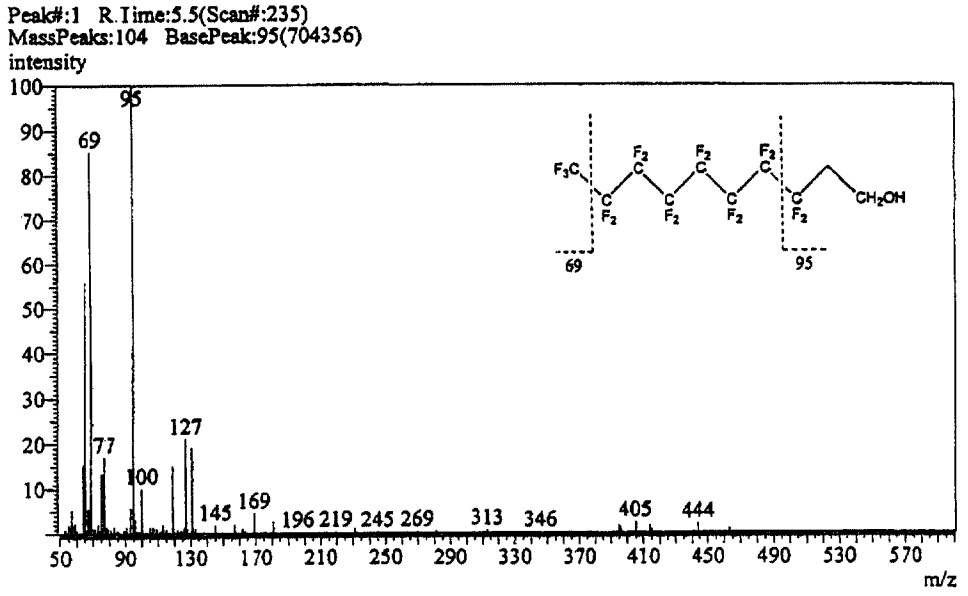
Fig.9 - 1 Mass spectrum of 2-(perfluorooctyl)ethanol (analytical condition).

Data file: 14114d001
Sample: standard solution 10 0mg/L

Total ion chromatogram



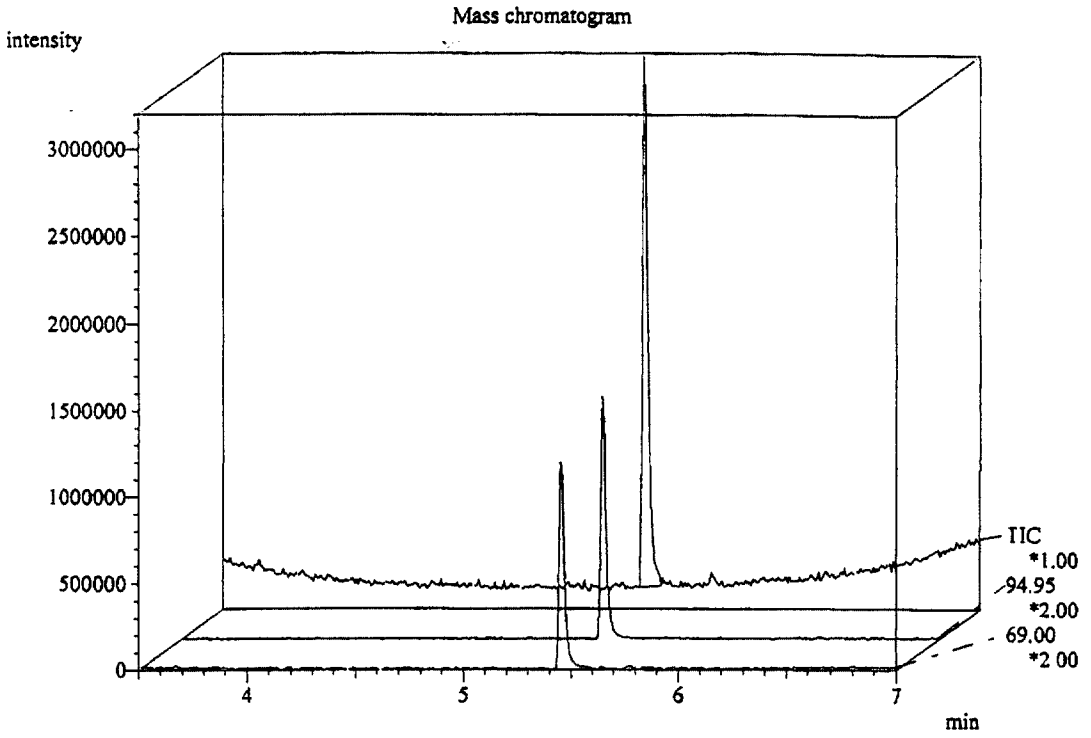
MS spectrum



Date : June 11, 2003 Name : K. Ojama

Fig.9 - 2 Mass spectrum of 2-(perfluorooctyl)ethanol (mass spectrum).

Data file: 14114d001
Sample: standard solution 10 0mg/L



Date : June 11, 2003 Name : K Dyamon

Fig.9 - 3 Mass spectrum of 2-(perfluorooctyl)ethanol (mass chromatogram)

Fig. 10-1 IR spectrum of test item measured before experimental start.

Study No. : 14114
Sample : Test item
Method : KBr tablet
Date : July 10, 2003
Name : K. Ojima

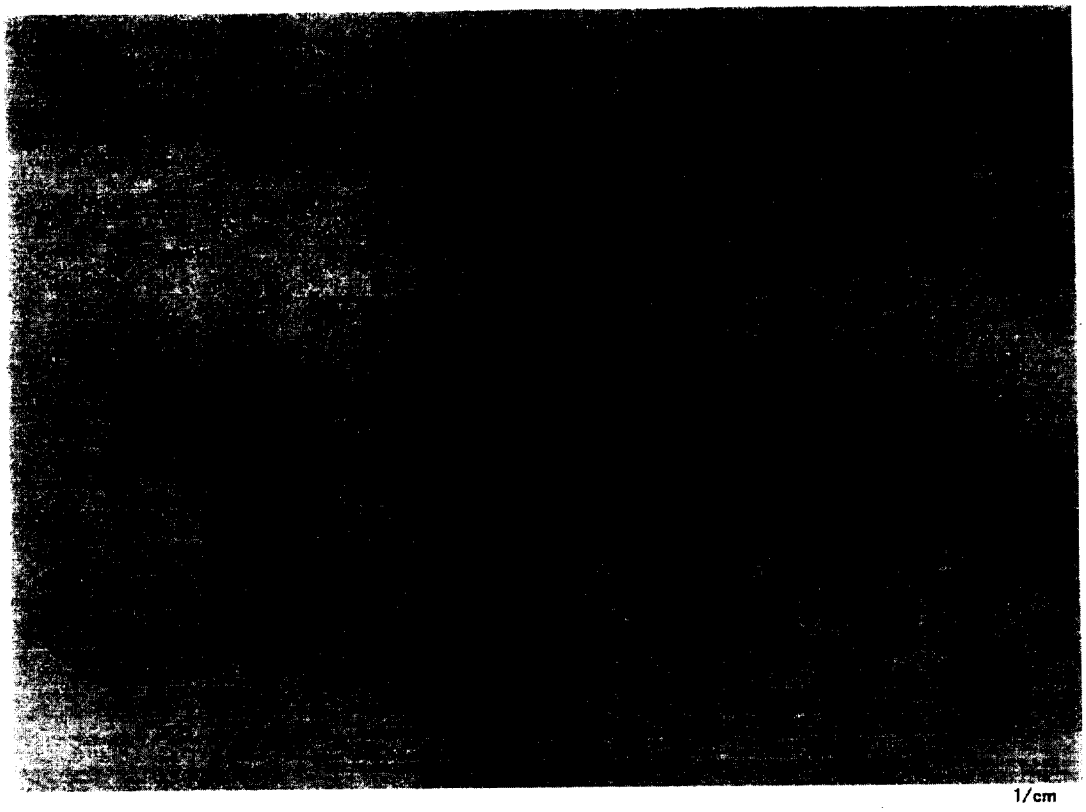


Fig. 10-2 IR spectrum of test item measured after experimental completion.

Study No. : 14114
Sample : Test item
Method : KBr tablet
Date : August 27, 2003
Name : K Ojima

Reference IR spectrum supplied by sponsor

Amendment to Final Report

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

1 Title — Biodegradation study of (CBI) by microorganisms

2. Study number — 14114

3. Content — 4.5 3 Recovery test and blank test (page 19)

4. Reason — Writing error

5. Content of correction

" Recovery rate of the test solutions (water + test item)
99 6 %, 98.2 % average 98.9 %
Recovery rate of the test solutions (sludge + test item)
80.5 %, 81.5 % average 81.0 % "

is corrected to

" Recovery rate of the test solutions (water + 2-(perfluorooctyl)ethanol)
99.6 %, 98.2 % average 98.9 %
Recovery rate of the test solutions (sludge + 2-(perfluorooctyl)ethanol)
80.5 %, 81.5 % average 81.0 % "

6. Approval

Date — October 10, 2003

Study Director — Signed in original

Yasuko Matsunobu

Accepted number	S03-4115
Study number	14115

FINAL REPORT

AR 226

Biodegradation study of (CBI) by microorganisms

September 18, 2003

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

Company Sanitized

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14115

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No.14115, issued on September 18, 2003, amended on October 10, 2003).

Date December 2, 2003

Study Director Y. Matsunobu
Yasuko Matsunobu

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14115

This study was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

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

Date September 18, 2003

Study Director Signed in original

Yasuko Matsunobu

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14115

Amendment to the Final Report was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997).

This GLP statement was issued as supplement to the statement issued on September 18, 2003 because of the amendment of the Final Report.

Date October 10, 2003

Study Director Signed in original

Yasuko Matsunobu

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14115

The inspections of this study were carried out and the results were reported to the test facility management and the Study Director by Quality Assurance Unit of Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan as follows.

Item of inspection	Date of inspection	Date of report to Study Director	Date of report to test facility management
Study plan	July 17, 2003	July 18, 2003	July 18, 2003
	August 11, 2003	August 11, 2003	August 11, 2003
	September 10, 2003	September 10, 2003	September 10, 2003
Test Conduct	July 18, 2003	July 18, 2003	July 18, 2003
	August 1, 2003	August 27, 2003	August 27, 2003
	August 15, 2003	August 27, 2003	August 27, 2003
	August 18, 2003	August 27, 2003	August 27, 2003
	August 19, 2003	August 27, 2003	August 27, 2003
	August 21, 2003	August 27, 2003	August 27, 2003
	August 26, 2003	August 27, 2003	August 27, 2003
Raw Data and Final Report	September 18, 2003	September 18, 2003	September 18, 2003

It has been assured that the final report describes accurately the test method used, that details in the report are in compliance with the study plan and Standard Operating Procedures and that the final report reflects the raw data accurately.

Date September 18, 2003

Quality Assurance Unit, Head Signed in original

Kyoshiro Hori

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title Biodegradation study of (CBI) by microorganisms

Study number 14115

Study inspection of the corrected parts in the Final Report was carried out and it was confirmed that the correction has no problem. The result was reported to the test facility management and the Study Director as follows.

Date of inspection	Date of report to Study Director	Date of report to test facility management
October 10, 2003	October 10, 2003	October 10, 2003

This statement was issued as a supplement to the quality assurance statement issued on September 18, 2003.

Date October 10, 2003

Quality Assurance Unit, Head Signed in original

Kyoshiro Hori

CONTENTS

Contents of tables and figures

Contents of tables

Contents of figures

Study No. 14115

Title

Biodegradation study of (CBI) by microorganisms

Sponsor

DAIKIN INDUSTRIES, LTD.
1-1 Nishi-hitotsuya, Settsu, Osaka 566-8585, Japan

Test facility

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan
19-14 Chuomachi, Kurume, Fukuoka 830-0023, Japan

Objective

This study was performed to evaluate the biodegradability of (CBI) by microorganisms.

Test method

This study was performed according to "Ready Biodegradability: Modified MITI Test (I) (Guideline 301C, Revised July 17, 1992)" in the OECD Guideline for Testing of Chemicals.

Applied GLP

This study was performed in compliance with "OECD Principles of Good Laboratory Practice" (November 26, 1997)

Dates

Study initiation date	July 17, 2003
Experimental starting date	July 18, 2003
Experimental completion date	August 15, 2003
Study completion date	September 18, 2003

Storage of test item, raw data, etc.

(1) Test item

About 5 g of the item supplied by the sponsor is sealed in a store vessel and stored in archives in this laboratory for ten years after the publication of the final report. Treatment of the item supplied by the sponsor after the storage period is discussed with sponsor. If it is not stable for the storage period, it is stored while it is kept stable and it is disposed with approval of sponsor.

(2) Raw data and materials, etc.

Raw data, the study plan, documents about the study presented by the sponsor, the final report and necessary materials are stored in archives in this laboratory for the same term as the test item. Treatment of raw data and materials, etc. after the storage period is discussed with sponsor.

Personnel

Study Director	Yasuko Matsunobu
Study personnel (Operation of biodegradation test)	Kazuhiro Oyama Takakazu Kayashima
Staff for cultivation of activated sludge	Hiroto Nishijima

Approval of final report

Study Director	Date	September 18, 2003
	Signature	Signed in original
		Yasuko Matsunobu

SUMMARY

Title

Biodegradation study of (CBI) by microorganisms

Conditions of cultivation

(1) Concentration of test item	100 mg/L
(2) Concentration of activated sludge (as the concentration of suspended solid)	30 mg/L
(3) Volume of test solution	300 mL
(4) Cultivation temperature	25±1 °C
(5) Cultivation duration	28 days

Measurement and analysis for percentage biodegradation

(1) Measurement of biochemical oxygen demand (BOD) by means of a closed system oxygen consumption measuring apparatus
(2) Determination of test item by means of weight measurement

Other measurement and analysis

(1) Determination of dissolved organic carbon by means of total organic carbon (TOC) analysis
(2) Measurement of IR spectrum by means of a fourier transform infrared spectrophotometer
(3) Determination of perfluorooctanoic acid by means of liquid chromatography-mass spectrometry
(4) Determination of 2-(perfluorooctyl)ethanol by means of gas chromatography-mass spectrometry

Results

(1) Percentage biodegradation by BOD	0 %,	0 %,	0 %	average 0 %
(2) Percentage biodegradation by weight measurement	0 %,	0 %,	0 %	average 0 %

Conclusion

The test item was not biodegraded by microorganisms under the present test conditions.

1. Test item

In this report, (CBI) has the following chemical name, etc.

1.1 Chemical name*1

(CBI)

1.2 Chemical structure, etc.*1

Structural formula

Molecular weight Weight-average (CBI)

*1 Information supplied by the sponsor

2. Item supplied by sponsor

2.1 Supplier and lot number*1

(1) Supplier DAIKIN INDUSTRIES, LTD.

(2) Lot number (CBI)

2.2 Purity*1

(1) Test item (CBI)

(2) Impurity (CBI)

The test item was treated as 100 % in purity.

2.3 Confirmation of test item

Two infrared (IR) spectra of the test item provided by the sponsor and measured at this laboratory were confirmed to be identical (see Fig.10 and Reference).

2.4 Physicochemical property*1

Appearance (CBI)

*1 Information supplied by the sponsor

2.5 Storage and stability

(1) Storage condition

Dark storage place at room temperature

(2) Stability

The test item was stable under the storage conditions, as shown by the finding that IR spectra of the test item before the experimental start and after the experimental completion were identical (see Fig.10).

3. Activated sludge

3.1 Sludge sampling sites and date

(1) Sampling sites

On-site sludge sampling was carried out at the following 10 locations in Japan.

Fushikogawa city sewage plant (Sapporo-shi, Hokkaido)
Fukashiba industrial sewage plant (Kashima-gun, Ibaraki)
Nakahama city sewage plant (Osaka-shi, Osaka)
Ochiai city sewage plant (Shinjuku-ku, Tokyo)
Kitakami River (Ishinomaki-shi, Miyagi)
Shinano River (Niigata-shi, Niigata)
Yoshino River (Tokushima-shi, Tokushima)
Lake Biwa (Otsu-shi, Shiga)
Hiroshima Bay (Hiroshima-shi, Hiroshima)
Dookai Bay (Kitakyushu-shi, Fukuoka)

(2) Date June, 2003

3.2 Sludge sampling

(1) City sewage

Return sludges from sewage plants were collected.

(2) Rivers, lake and sea

Surface water and surface soil which was in contact with the atmosphere were collected.

3.3 Preparation of activated sludge

Activated sludge was prepared as follows to maintain its uniformity.

The filtrate (5 L) of the supernatant of the activated sludge*2 cultivated about for 3 months was mixed with the mixed filtrate (5 L) of the supernatant of a sludge collected newly at each location. The mixed filtrate (10 L) was aerated*3 after the pH value of the mixture was adjusted to 7.0±1.0.

*2 The activated sludge cultivated the mixed filtrate (10 L) of the supernatant of sludge collected at the ten locations.

*3 Prefiltered open air was used

3.4 Cultivation

Roughly 30 minutes after ceasing aeration of the sludge mixture, supernatant corresponding to about 1/3 of the whole volume was removed. Dechlorinated water was added to the remaining portion so that the total volume reached 10 L. This mixture was aerated, and then a predetermined amount of synthetic sewage*4 was added to the mixture so that the concentration of the synthetic sewage was 0.1 wt% in the volume of dechlorinated water added. This procedure was repeated once every day. Cultivation was carried out at 25±2 °C.

*4 Synthetic sewage

Glucose, peptone and potassium dihydrogenphosphate were dissolved in purified water to obtain 50 g/L of the solution for each component. The pH of the solution was adjusted to 7.0±1.0 with sodium hydroxide.

3.5 Control and use

During cultivation, the appearance of the supernatant, sedimentation of the sludge, formation of flock, pH, dissolved oxygen concentration in the solution and temperature were checked to maintain a normal state of sludge. It was confirmed that these were within the scope of the control standard stipulated in the "Testing Methods for New Chemical Substances", and these results were stored as raw data. Microflora in the activated sludge was microscopically observed and sludge with no abnormal symptoms was used for the test.

3.6 Inspection of activity and date of initiation of use of activated sludge

(1) Inspection of activity

Activity of the sludge was assessed using a reference item.

(2) Date of initiation of use July 15, 2003

4. Performance of biodegradation test

4.1 Preparations for test

(1) Measurement of concentration of suspended solid

The concentration of suspended solid was measured to determine the amount of activated sludge to add.

Method	In accordance with Japanese Industrial Standards (JIS) K 0102-1998 section 14.1
Date	July 15, 2003
Result	Concentration of suspended solid in the activated sludge was 3200 mg/L.

(2) Preparation of basal culture medium

Each 3 mL of solutions (a), (b), (c) and (d) shown below were made up to 1L with purified water.

The following stock solutions were prepared by use of analytical grade regents:

(a) Potassium dihydrogenphosphate, KH_2PO_4 8.50 g
Dipotassium hydrogenphosphate, K_2HPO_4 21.75 g
Disodium hydrogenphosphate dodecahydrate, $Na_2HPO_4 \cdot 12H_2O$ 44.60 g
Ammonium chloride, NH_4Cl 1.70 g
Dissolved in water and filled up to 1 L

(b) Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$ 22.50 g
Dissolved in water and filled up to 1 L

(c) Calcium chloride anhydrous, $CaCl_2$ 27.50 g
Dissolved in water and filled up to 1 L

(d) Iron(III) chloride hexahydrate, $FeCl3 \cdot 6H_2O$ 0.25 g
Dissolved in water and filled up to 1 L

(3) Reference item

Aniline (reagent grade, Showa Chemicals Inc. Lot No. HO-2729D) was used as a reference item to confirm that the sludge was sufficiently active.

This test is effective when the percentage biodegradation calculated from the BOD of aniline after 7 and 14 days exceeds 40 % and 65 %, respectively, according to regulation of the OECD Guideline for Testing of Chemicals.

4.2 Preparation of test solutions

Test solutions for analysis of the test item and perfluorooctanoic acid and test solutions for analysis of 2-(perfluorooctyl)ethanol were prepared, because the test item and converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected to form in test solutions, could not be analyzed by the same pretreatment.

Eleven test vessels were prepared. The following test solutions were prepared and cultured under the conditions described in section 4.3.

(1) Addition of test item or aniline

(a) Test solution (water + test item)

(n=2: Vessel No.6 and a test solution for analysis of 2-(perfluorooctyl)ethanol)

In one test vessel, 30 mg of the item supplied by the sponsor was accurately weighed and added to 300 mL of purified water, so that the concentration of the test item reached 100 mg/L.

(b) Test solution (sludge + test item)

(n=6: Vessel No.1, 2, 3 and three of test solutions for analysis of 2-(perfluorooctyl)ethanol)

In each test vessel, 30 mg of the item supplied by the sponsor was accurately weighed and added to the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated), so that the concentration of the test item reached 100 mg/L.

(c) Test solution (sludge + aniline) (n=1: Vessel No.4)

In one test vessel, 29.5 μL [30 mg = 29.5 μL × 1.022 g/cm^3 (density)] of aniline was added into the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated), so that the concentration reached 100 mg/L.

(d) Test solution (control blank)

(n=2: Vessel No.5 and a test solution for analysis of 2-(perfluorooctyl)ethanol)

In one test vessel, nothing was added to the basal culture medium (the volume was less than 300 mL by the volume (2.81 mL) of activated sludge inoculated).

(2) Inoculation of activated sludge

The activated sludge cultivated under the conditions described in section 3 was added to each test vessel, (b), (c) and (d), so that the concentration of the suspended solid reached 30 mg/L.

4.3 Instruments and conditions of cultivation

(1) Instruments for cultivation

(a) Test solution for analysis of test item and perfluorooctanoic acid

Closed system oxygen consumption measuring apparatus

Temperature controlled bath and measuring unit :
Ohkura Electric Co., Ltd.

Data sampler : Asahi Techneion Co., Ltd.

Vessel 300 mL in volume (improved type)

Absorbent for carbon dioxide

Soda lime No.1 (for absorption of carbon dioxide,
Wako Pure Chemical Industries, Ltd.)

(b) Test solution for analysis of 2-(perfluorooctyl)ethanol

Closed system oxygen consumption measuring apparatus

Temperature controlled bath and measuring unit
(Only the temperature controlled bath was used) :
Ohkura Electric Co., Ltd.

Vessel 300 mL in volume (sealed improved type)

(2) Conditions of cultivation

Cultivation temperature	25±1 °C
Cultivation duration	28 days
Stirring method	Each test solution was stirred by a magnetic stirrer.

(3) Room Apparatus room No.511

4.4 Observation and measurement of test conditions

(1) Observation of test solution

During the cultivation period, the appearance of the test solution was observed periodically and conditions of the instruments were checked properly.

(2) Measurement of biochemical oxygen demand (BOD)

During the cultivation period, the change in BOD of the test solutions was measured by autorecording using a data sampler. Cultivation temperature was measured and recorded once a day. BOD of the test solutions for analysis of 2-(perfluorooctyl)ethanol was not measured.

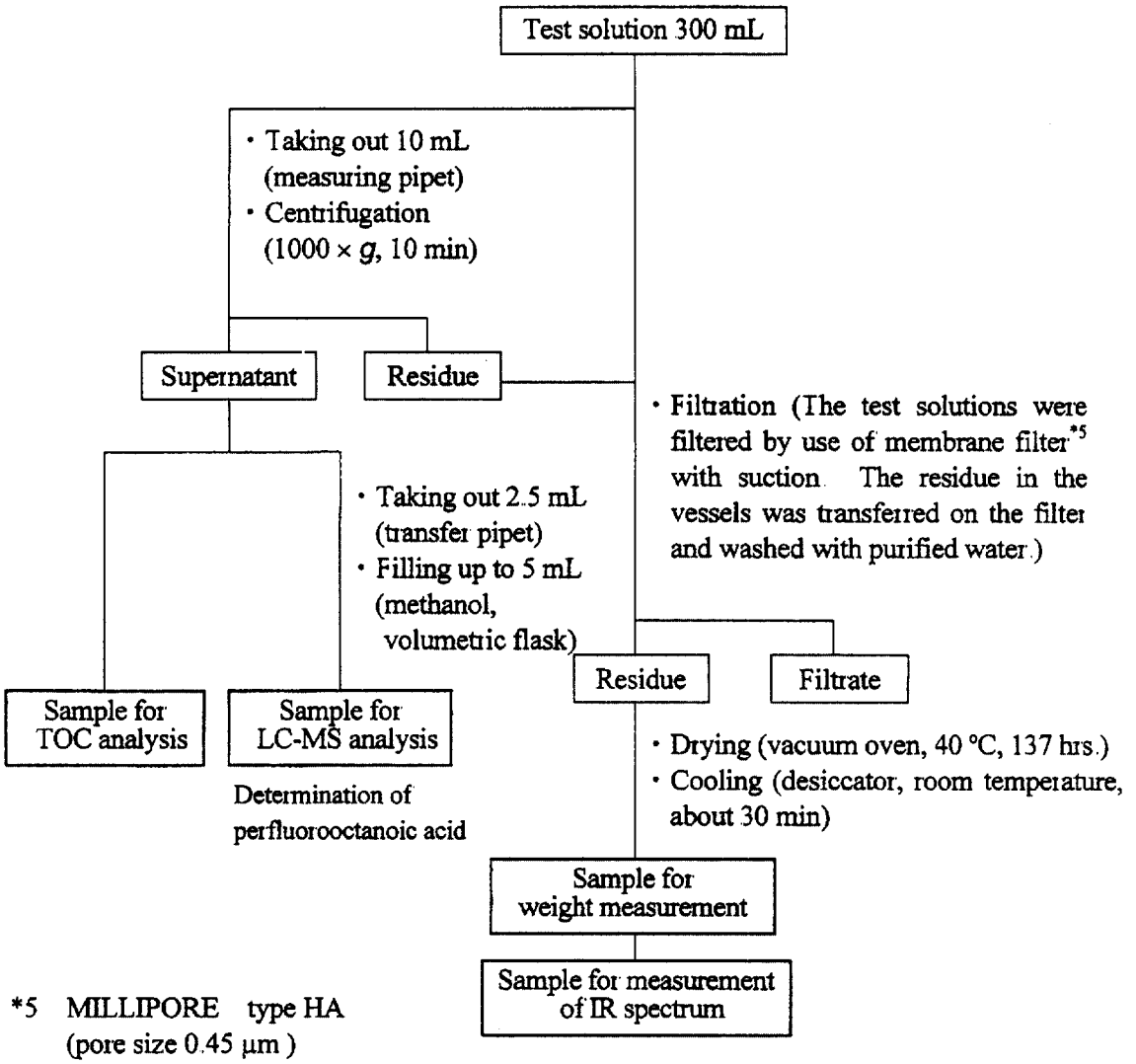
4.5 Analysis of test solution

After the termination of the cultivation, dissolved organic carbon, the test item and converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected production in test solutions, were determined.

4.5.1 Pretreatment of test solutions for analysis

(1) Pretreatment for analysis of test item and perfluorooctanoic acid

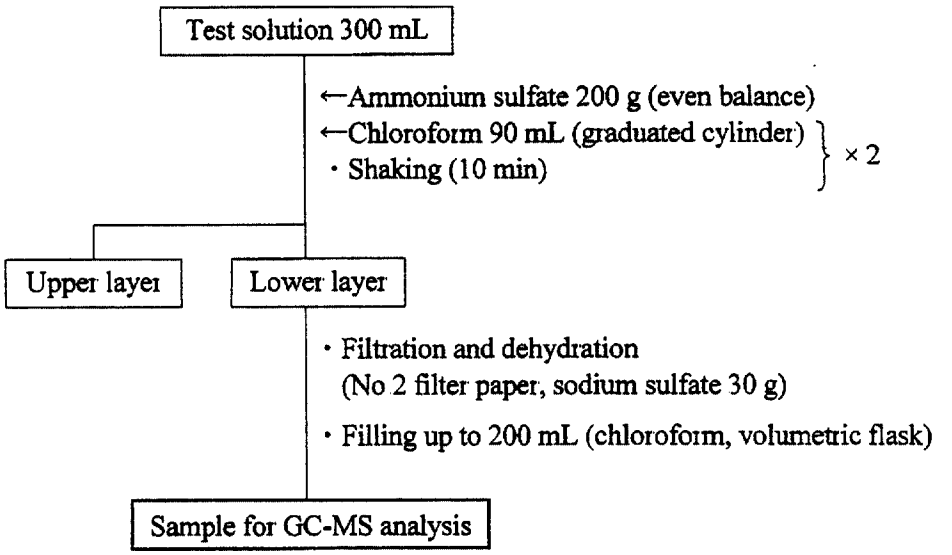
After the termination of the cultivation, the test solution (water + test item), the test solutions (sludge + test item) and the test solution (control blank) for analysis of the test item and perfluorooctanoic acid were pretreated for total organic carbon (TOC) analysis on dissolved organic carbon and liquid chromatography - mass spectrometry (LC-MS) analysis of perfluorooctanoic acid as follows. Weight measurement of the test item was performed. IR spectrum of the residue was measured.



*5 MILLIPORE type HA (pore size 0.45 μm)

(2) Pretreatment for analysis of 2-(perfluorooctyl)ethanol

After the termination of the cultivation, the test solution (water + test item), the test solutions (sludge + test item) and the test solution (control blank) for analysis of 2-(perfluorooctyl)ethanol were cooled at 5 ℃ for 66 hrs. and pretreated for gas chromatography - mass spectrometry (GC-MS) analysis as follows.



4.5.2 Quantitative and qualitative analysis

(1) Determination of dissolved organic carbon by means of TOC analysis

The dissolved organic carbon (DOC) in the samples for TOC analysis was analyzed under the following conditions.

The concentration of DOC was calculated by subtracting concentration of the inorganic carbon (IC) from concentration of the total carbon (TC). The concentration of TC and IC in the test solutions was proportionally calculated from the peak area of the test solution by comparison with that of 80.0 mgC/L of a standard solution for TC analysis and 80.0 mgC/L of a standard solution for IC analysis, respectively (see Table-2).

The standard solution for TC analysis was prepared by dissolving potassium hydrogenphthalate in purified water. The standard solution for IC analysis was prepared by dissolving sodium hydrogencarbonate and sodium carbonate in purified water.

The concentration of dissolved organic carbon corresponding to the minimum determination limit was regarded as 1.0 mgC/L.

Analytical conditions

Instrument	Total organic carbon analyzer Shimadzu Corporation type TOC-5000
Temperature of furnace	680 °C
Flow rate	150 mL/min
Injection volume	33 μL
Sensitivity	Range 5

(2) Determination of test item by means of weight measurement

Weight of the samples for weight measurement was measured under the following conditions (see Table-3).

Analytical conditions

Instrument	Electronic analytical balance Mettler Toledo type AT201
Minimum measuring weight	0.1 mg

(3) Measurement of IR spectrum by means of fourier transform infrared spectrophotometer

IR spectrum of the samples for measurement of IR spectrum was measured under the following conditions (see Fig.2).

Analytical conditions

Instrument	Fourier transform infrared spectrophotometer Shimadzu Corporation type FTIR-8200PC
Measuring method	KBr tablet

(4) Determination of perfluorooctanoic acid by means of LC-MS

The samples for LC-MS analysis were analyzed for perfluorooctanoic acid under the following conditions. The concentration of perfluorooctanoic acid in the sample for LC-MS analysis was proportionally calculated by comparing the peak area on the mass fragmentogram of the sample for LC-MS analysis with that on the mass fragmentogram of 0.500 mg/L standard solution (see Table-4 and Fig.6).

The lowest detectable peak area was regarded as 2000 considering the noise level, which corresponded to 0.0097 mg/L of perfluorooctanoic acid.

(a) Analytical conditions

Instrument	Liquid chromatograph–mass spectrometer
LC	JASCO Corporation type PU-980
MS	Micromass type Quattro II

Conditions of LC

Column	L-column ODS 15 cm × 4.6 mm I.D.
Eluent	Methanol[*6] / purified water[*6] (80/20 V/V)
Flow rate	1.0 mL/min
Sample size	10 μL

Conditions of MS

Ionization method	Electrospray (ESI)
Detection mode	Negative ion
Monitoring method	Selected ion monitoring (SIM)
Monitoring m/z	413 (see Fig.8)
Source temperature	150 ℃
Cone voltage	20 V

*6 Containing 0.1 % formic acid

(b) Preparation of standard solution

The standard solution to determine the concentration of perfluorooctanoic acid in the sample for LC-MS analysis was prepared as follows.

100 mg of the standard substance*7 supplied by the sponsor was accurately weighed and dissolved in methanol to obtain 2000 mg/L solution of perfluorooctanoic acid. 0.500 mg/L standard solution was then prepared from this solution by dilution with methanol / purified water (1/1 V/V).

*7 Name, lot number and purity

Name	Perfluorooctanoic acid (C-1700)
Lot number	C17001401
Purity	Perfluorooctanoic acid was treated as 100 % in purity.

(c) Calibration curve

0.125, 0.250 and 0.500 mg/L standard solutions were prepared by the same method as described in (b). These solutions were analyzed according to the analytical conditions described in (a). A calibration curve was drawn based on the relation between the peak area on the mass fragmentograms and the respective concentrations (see Fig.3).

(5) Determination of 2-(perfluorooctyl)ethanol by means of GC-MS

The samples for GC-MS analysis were analyzed for 2-(perfluorooctyl)ethanol under the following conditions. The concentration of 2-(perfluorooctyl)ethanol in the sample for GC-MS analysis was proportionally calculated by comparing the peak area on the mass fragmentogram of the sample for GC-MS analysis with that on the mass fragmentogram of 0.500 mg/L standard solution (see Table-6 and Fig.7).

The lowest detectable peak area was regarded as 300 considering the noise level, which corresponded to 0.021 mg/L of 2-(perfluorooctyl)ethanol.

(a) Analytical conditions

Instrument	Gas chromatograph–mass spectrometer Shimadzu Corporation type QP-5000

Conditions of GC

Column	HP-FFAP (Agilent) 25 m × 0.32 mm I.D., made of fused silica
Column temperature	50 °C (4 min)→170 °C (Rate 30 °C/min)
Carrier gas	Helium
Pressure of carrier gas	10.0 kPa
Injection temperature	200 °C
Sample size	2 μL
Sampling method	Splitless
Sampling time	2.0 min

Conditions of MS

Ionization method	Electron ionization (EI)
Detection mode	Positive ion
Monitoring method	Selected ion monitoring (SIM)
Monitoring m/z	95 (see Fig.9)
Interface temperature	230 °C

(b) Preparation of standard solution

The standard solution to determine the concentration of 2-(perfluorooctyl)-ethanol in the sample for GC-MS analysis was prepared as follows.

100 mg of the standard substance[*8] supplied by the sponsor was taken out accurately and dissolved in chloroform to obtain 2000 mg/L solution of 2-(perfluorooctyl)ethanol. 1.50 mg/L standard solution was then prepared from this solution by dilution with chloroform.

*8 Name, lot number and purity

Name	2-(perfluorooctyl)ethanol (A-1280)
Lot number	A18201X01
Purity	2-(perfluorooctyl)ethanol was treated as 100 % in purity.

(c) Calibration curve

0.375, 0.750 and 1.50 mg/L standard solutions were prepared by the same method as described in (b). These solutions were analyzed according to the analytical conditions described in (a). A calibration curve was drawn based on the relation between the peak area on the mass fragmentograms and the respective concentrations (see Fig.4).

4.5.3 Recovery test and blank test

Each two test solutions (water + 2-(perfluorooctyl)ethanol) and two test solutions (sludge + 2-(perfluorooctyl)ethanol) for recovery test were prepared according to the methods described in section 4.2. 0.300 mg of 2-(perfluorooctyl)ethanol was added to each test vessel, so that the concentration of it reached 1 mg/L. The test solutions were pretreated in accordance with the method described in section 4.5.1(2), then analyzed according to analytical conditions described in section 4.5.2(5).

A test solution for blank test was prepared according to the method described in section 4.2. The test solution for blank test was analyzed in the same way as the recovery test. As for the blank test, no peaks were appeared around the peak of the 2-(perfluorooctyl)ethanol on the mass fragmentogram.

Two individual recovery rates and their averages on the analytical procedure are shown below. The average recovery rates were used as correction factor, for the determination of 2-(perfluorooctyl)ethanol in the test solutions (see Table-5 and Fig.5).

Recovery rate of the test solutions (water + 2-(perfluorooctyl)ethanol)
99.6 %, 98.2 % average 98.9 %

Recovery rate of the test solutions (sludge + 2-(perfluorooctyl)ethanol)
80.5 %, 81.5 % average 81.0 %

Corrected page

4.6 Calculation of percentage biodegradation

The percentage biodegradation was calculated by the following equations and expressed in whole numbers.

(1) Percentage biodegradation by BOD

$$\text{Percentage biodegradation (\%)} = \frac{\text{BOD} - \text{B}}{\text{TOD}^{*9}} \times 100$$

BOD	:	Biochemical oxygen demand in the test solution (sludge + test item) (experimental) (mg)
B	:	Biochemical oxygen demand in the control blank (experimental) (mg)
TOD^{*9}	:	Theoretical oxygen demand required when the test item was completely oxidized (theoretical) (mg)

*9 The purity was regarded as 100 % and TOD was calculated from composition formula (CBI) which was calculated by use of n=8, monomer values of (CBI) regarded as mol% in section 1.2.

(2) Percentage biodegradation by weight measurement

$$\text{Percentage biodegradation (\%)} = \frac{\text{Sw} - \text{Ss}}{\text{Sw}} \times 100$$

Ss	:	Residual amount of the test item in the test solution (sludge + test item) (experimental) (mg)
Sw	:	Residual amount of the test item in the test solution (water + test item) (experimental) (mg)

4.7 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401:1999 rule B.

5. Validity of test conditions

Percentage biodegradations of aniline calculated by the BOD values were 65 % and 69 % after 7 and 14 days, respectively. It was concluded that this test conditions were valid (see Table-1 and Fig.1).

6. Factors possibly affecting accuracy

No adverse effects on the reliability of this test were noted.

7. Results

7.1 Appearances of test solutions

Appearances of test media in cultivation vessels were as follows.

	Test solution	Appearance	pH
At the start of cultivation	Water + test item	The test item was not dissolved.	-
	Sludge + test item	The test item was not dissolved.	-
At the termination of cultivation	Water + test item	Insoluble compound was observed.	-
	Sludge + test item	Insoluble compound except for the sludge was observed. Growth of the sludge was not observed.	-

7.2 Analytic results of test solutions

Analytic results of the test solution after 28 days were as follows.

		Water + test item	Sludge + test item			Theoretical amount	Table	Fig.
		Vessel-6	Vessel-1	Vessel-2	Vessel-3			
BOD*10	mg	0	0.2	0	0	(CBI)	1	1
*10 Detection amount and percentage detection of DOC	mgC	0	0	0	0	(CBI)	2	-
	%	0	0	0	0	-		
Residual amount and percentage residue of test item (Weight measurement)	mg	28.2	30.1	30.8	29.2	30.0	3	-
	%	94	100	103	97	-		
*11 Concentration of perfluorooctanoic acid (LC-MS)	mg/L	≦0.019	≦0.019	≦0.019	≦0.019	-	4	6

		Water + test item	Sludge + test item			Theoretical amount	Table	Fig.
			1	2	3			
*12 Concentration of 2-(perfluorooctyl) ethanol (GC-MS)	mg/L	≦0.014	≦0.017	≦0.017	≦0.017	-	6	7

*10 The value of control blank was subtracted from the values of the test solutions (sludge + test item).

*11 Minimum determination limit of perfluorooctanoic acid in test solution
= (Minimum determination limit in sample for LC-MS analysis) ×
{(final volume) / (volume of test solution)} / (ratio of portion used for analysis)
= 0.0097 mg/L × (5 mL / 300 mL) / (2.5/300) = 0.019 mg/L

*12 Minimum determination limit of 2-(perfluorooctyl)ethanol in test solution
= (Minimum determination limit in sample for GC-MS analysis)×
{(final volume) / (volume of test solution)} / {(average recovery rate) / 100} /
(ratio of portion used for analysis)
Test solution (water + test item) :
0.021 mg/L × (200 mL / 300 mL) / (98.9/100) / 1 = 0.014 mg/L
Test solution (sludge + test item) :
0.021 mg/L × (200 mL / 300 mL) / (81.0/100) / 1 = 0.017 mg/L

7.3 Percentage biodegradation

Percentage biodegradations after 28 days were as follows

Method	Percentage biodegradation (%)				Table
	Vessel-1	Vessel-2	Vessel-3	Average	
BOD	0	0	0	0	1
Weight measurement	0	0	0	0	3

7.4 Results of measurement of IR spectrum

No significant difference between IR spectrum of the test item and that of the residue after weight measurement was observed (see Figs.2, 10).

7.5 Discussion

Determination of the test item by chromatography was difficult, because the test item was not completely dissolved in water and organic solvents (tetrahydrofuran and chroloform). Therefore, the test item was determined by means of weight measurement. As a result, the percentage residues of the test item were 94 % in the test solution (water + test item) and 100, 103 and 97 % in the test solutions (sludge + test item). The percentage detections of DOC were 0 % in the test solution (water + test item) and 0 % in all test solutions (sludge + test item). These results indicate that the test item was not dissolved in the test solution.

No significant difference between IR spectra of the test item before and after the cultivation was observed. Converted products (perfluorooctanoic acid and 2-(perfluorooctyl)ethanol), which was expected to form in test solutions, was not detected. These results indicate that the test item was not converted during the cultivation.

It is considered that the test item was not biodegraded during microorganisms, because the average percentage biodegradation by BOD was 0 % and no significant change in the weight of the test item before and after the cultivation was observed.

7.6 Conclusion

The test item was not biodegraded by microorganisms under the present test conditions.

8. Remarks

8.1 Instruments used for test

Fourier transform infrared spectrophotometer :
Shimadzu Corporation type FTIR-8200PC

Closed system oxygen consumption measuring apparatus :
see page 11

Total organic carbon analyzer : see page 14

Liquid chromatograph- mass spectrometer :
see page 15

Gass chromatograph- mass spectrometer :
see page 17

Electronic analytical balance : Mettler Toledo type AT201
Sartorius AG type CP324S
Sartorius AG type BP210S

Refrigerated centrifuge : Shimadzu Corporation type CST-060LF

Mechanical shaker : TIETECH Co., Ltd. type SR-2w

Vaccume oven : SIBATA SCIENTIFIC TECHNOLOGY LTD. type VS-300

8.2 Reagents used for analysis

Methanol (HPLC grade) : Wako Pure Chemical Industries, Ltd.

Purified water : Takasugi Seiyaku Co., Ltd.

Chloroform (reagent grade) : Kishida Chemical Co., Ltd.

Ammonium sulfate (reagent grade) : Kanto Chemical Co., Inc.

Sodium sulfate (reagent grade) : Kanto Chemical Co., Inc.

Formic acid (reagent grade) : Wako Pure Chemical Industries, Ltd.

Sodium hydrogencarbonate (reagent grade) :
Wako Pure Chemical Industries, Ltd.

Sodium carbonate (reagent grade) : Wako Pure Chemical Industries, Ltd.

Potassium hydrogenphthalate (reagent grade) :
Wako Pure Chemical Industries, Ltd.

Potassium bromide (for measurement of infrared absorption) :
NACALAI TESQUE, INC.

Perfluorooctanoic acid : Standard substance supplied by the sponsor

2-(perfluorooctyl)ethanol : Standard substance supplied by the sponsor

Table 1
Calculation table for the percentage biodegradation by BOD.

Test No. 14115 Cultivating Duration: 28 days

Vessel No.	7th day		14th day		21st day		28th day		Mean Deg. %
	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	BOD (mg)	Deg. (%)	
4	61.9	65	67.0	69	68.9	70	70.0	70	
5	2.9	-	4.8	-	6.0	-	7.2	-	
1	2.8	0	4.4	0	5.8	0	7.4	0	0
2	2.1	0	3.6	0	5.4	0	7.0	0	
3	3.1	0	4.7	0	5.9	0	7.1	0	
6	0.0	-	0.0	-	0.0	-	0.0	-	

Deg. : Percentage biodegradation

Vessel no. 4 : Sludge + Aniline
Vessel no. 5 : Control blank [B]
Vessel no. 1 2 3 : Sludge + Test substance
Vessel no. 6 : Water + Test substance

Test substance of 30 mg was added.

Chart of BOD : Fig. 1

Deg. = [BOD - B] / [TOD] × 100 (%)

TOD of test substance : (CBI) (mg)

[redacted]

TOD = 30.0 × (CBI) = (CBI) (mg)

TOD of aniline : 90.3 (mg)

$C_6H_7N + 8.75\ O_2 \rightarrow 6\ CO_2 + 3.5\ H_2O + NO_2$

$8.75\ O_2 / C_6H_7N = 279.99 / 93.13 = 3.01$

TOD = 30.0 × 3.01 = 90.3 (mg)

2003.08.15 Name k. Ogawa

Table-2 Calculation table for amount of dissolved organic carbon by TOC

Study No. 14115

Sample description	A	B	C	D
Water blank	n.d.			
[6] Water + test item	n.d.	0	0	0
[1] Sludge + test item	n.d.	0	0	
[2] Sludge + test item	1.01	0	0	0
[3] Sludge + test item	1.02	0	0	
[5] Control blank	1.14			

Amount of test item added : 30 (mg)

Volume of test solution : 300 (mL)

Theoretical amount of carbon :(CBI) (mgC) (30) × (CBI)

A : Measured value (mgC/L)

B : Amount of DOC

Bw = (A(Water + test item) - A(Water blank)) × 300 / 1000 (mgC)

Bs = (A(Sludge + test item) - A(Control blank)) × 300 / 1000 (mgC)

C : Percentage detection (%)

C = B / (Theoretical amount of carbon) × 100 (%)

D : Average percentage detection (%)

September 3, 2003 Name k. Ogawa

Table-3 Calculation table for percentage biodegradation by weight measurement

Study No. 14115

Sample description	A	B	C	D	E
[6] Water + test item	28.2	28.2	94		
[1] Sludge + test item	33.7	30.1	100	0	
[2] Sludge + test item	34.4	30.8	103	0	0
[3] Sludge + test item	32.8	29.2	97	0	
[5] Control blank	3.6				

Amount of test item added : 30 (mg)

Volume of test solution : 300 (mL)

A : Weight of residue (mg)

B : Residual amount of test item (mg)

Bw = A(Water + test item)

Bs = A(Sludge + test item) - A(Control blank)

C : Percentage residue (%)

C = B / (Amount of test item added) × 100

D : Percentage biodegradation (%)

D = (Bw - Bs) / Bw × 100

E : Average percentage biodegradation (%)

September 3, 2003 Name K. Oyama

Table-4 Calculation table for concentration of perfluorooctanoic acid by LC-MS

Study No. 14115

Sample description	A	D	E
Standard solution 0.500mg/L	103449		
[6] Water + test item	n.d.	0 (≦0.019)	-
[1] Sludge + test item	n.d.	0 (≦0.019)	
[2] Sludge + test item	n.d.	0 (≦0.019)	-
[3] Sludge + test item	n.d.	0 (≦0.019)	
[5] Control blank	n.d.		

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 5 (mL)

C : Ratio of portion used for analysis : 2.5/300

D : Concentration of perfluorooctanoic acid in test water (mg/L)

$D = F \times (A / A(\text{Standard})) \times (B / 300) / C$

Minimum determination limit : 0.019 (mg/L)

E : Average concentration of perfluorooctanoic acid (mg/L)

F : Concentration of standard solution : 0.500 (mg/L)

See Fig. 6

September 18, 2003 Name K. Ozawa

Table-5 Calculation table for recovery rate by GC-MS
(2-(perfluorooctyl)ethanol)

Study No. 14115

Sample description	A	D	E	F
Standard solution 1.50mg/L	26329			
Water + 2-(perfluorooctyl)ethanol -1	26222	0.299	99.6	98.9
Water + 2-(perfluorooctyl)ethanol -2	25861	0.295	98.2	
Sludge + 2-(perfluorooctyl)ethanol -1	21196	0.242	80.5	81.0
Sludge + 2-(perfluorooctyl)ethanol -2	21459	0.245	81.5	
Control blank	n.d.			

Amount of 2-(perfluorooctyl)ethanol added : 0.300 (mg)

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 200 (mL)

C : Ratio of portion used for analysis : 1

D : Recovery amount (mg)

$D = G \times (A / A(\text{Standard})) \times (B / 300) / C$

E : Recovery rate (%)

$E = D / 0.300 \text{ (mg)} \times 100$

F : Average recovery rate (%)

See Fig. 5

September 11, 2003 Name K. Ozawa

Table-6 Calculation table for concentration of 2-(perfluorooctyl)ethanol by GC-MS

Study No. 14115

Sample description	A	E	F
Standard solution 1.50mg/L	21672		
Water + test item	n.d.	0 (≦0.014)	-
Sludge + test item -1	n.d.	0 (≦0.017)	
Sludge + test item -2	n.d.	0 (≦0.017)	-
Sludge + test item -3	n.d.	0 (≦0.017)	
Control blank	n.d.		

Volume of test solution : 300 (mL)

A : Peak area (-)

B : Final volume : 200 (mL)

C : Ratio of portion used for analysis : 1

D : Recovery rate : 98.9 (%) (Water + test item)

81.0 (%) (Sludge + test item)

E : Concentration of 2-(perfluorooctyl)ethanol in test solution (mg/L)

$E = G \times (A / A(\text{Standard})) \times (B / 300) / (D / 100) / C$

Minimum determination limit : 0.014 (mg/L) (Water + test item)

: 0.017 (mg/L) (Sludge + test item)

F : Average concentration of 2-(perfluorooctyl)ethanol (mg/L)

G : Concentration of standard solution : 1.50 (mg/L)

See Fig. 7

September 18, 2003 Name K. Ogawa

Fig.1 Chart of BOD

Test No. 14115 (Test substance CBI)

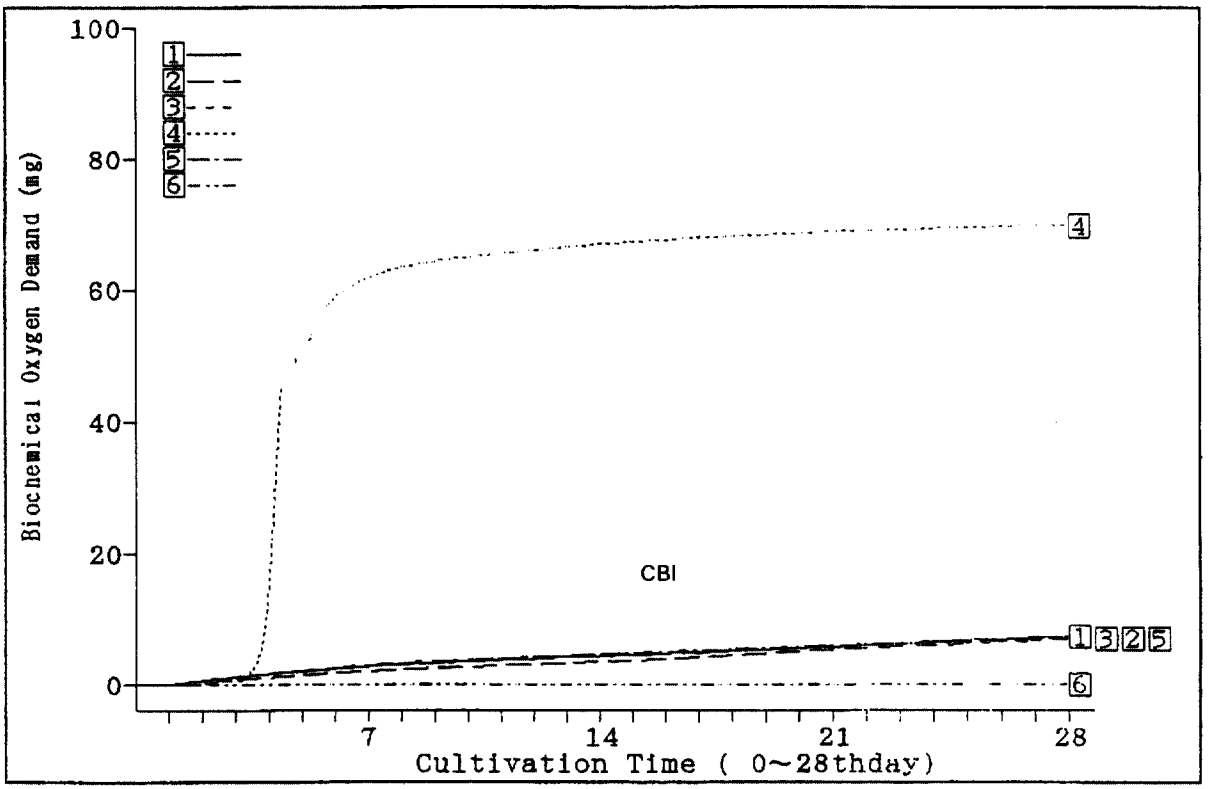
Apparatus No. CM-23

Cultivating conditions:

Concentration	
Test substance	100 (mg/ℓ)
Reference substance(aniline)	100 (mg/ℓ)
Activated sludge	30 (mg/ℓ)
Temperature	25 ± 1° C
Duration	28days(Jul.18~Aug 15,2003)

Note: Regular condition

Vessel no.	Sample description	BOD (mg)			
		7thday	14thday	21stday	28thday
1	Sludge + Test substance	2.8	4.4	5.8	7.4
2	Sludge + Test substance	2.1	3.6	5.4	7.0
3	Sludge + Test substance	3.1	4.7	5.9	7.1
4	Sludge + Aniline	61.9	67.0	68.9	70.0
5	Control blank [B]	2.9	4.8	6.0	7.2
6	Water + Test substance	0.0	0.0	0.0	0.0



2003.08.15 Name K. Ogama

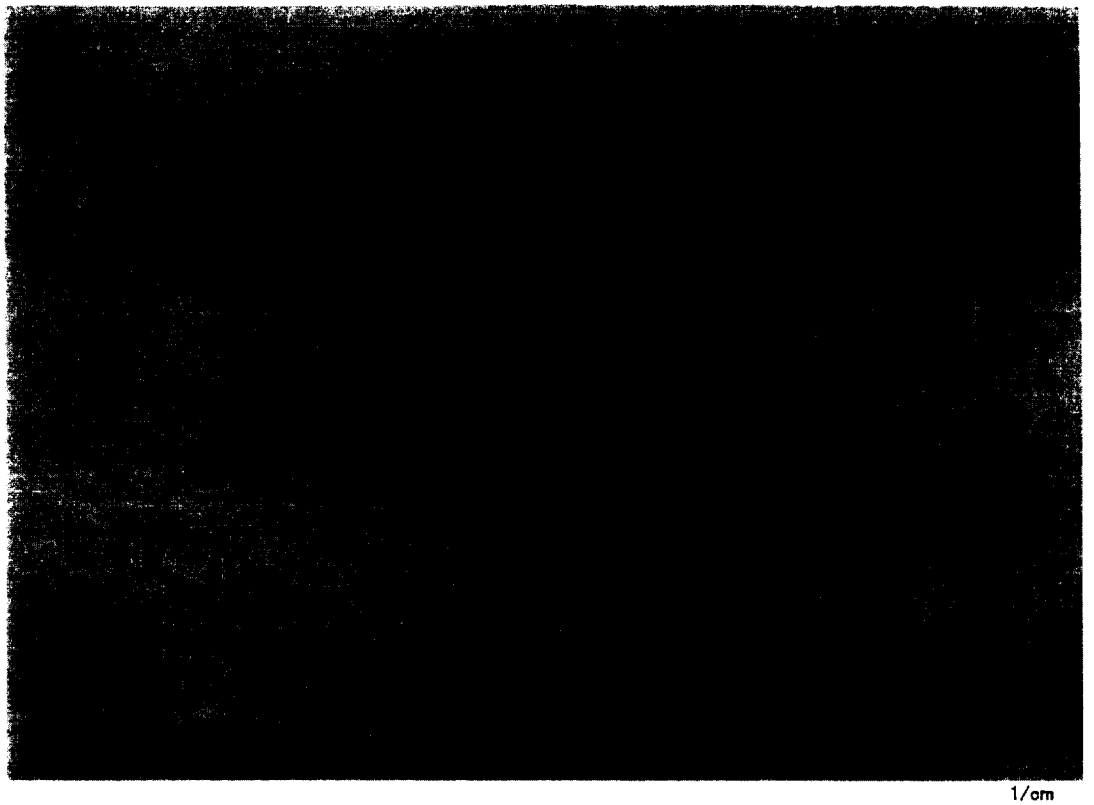


Fig. 2 -1 IR spectrum of residue.

Study No. : 14115
Sample : [6] Water + test item
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

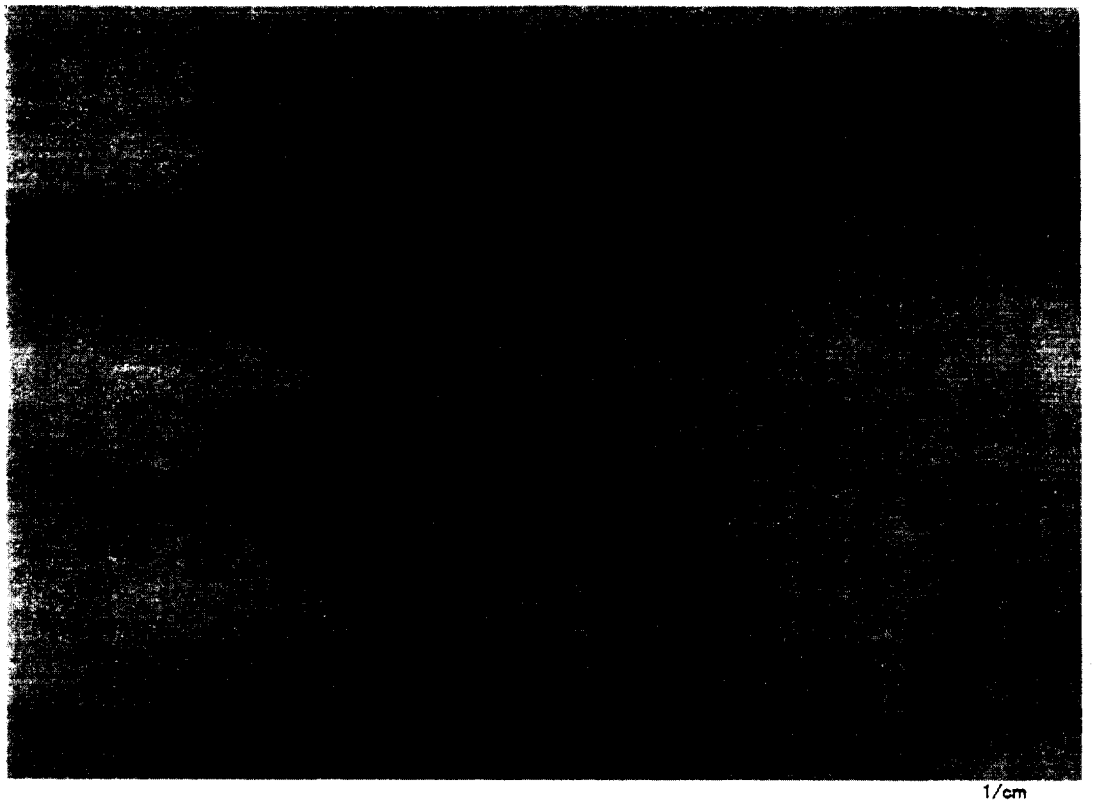


Fig 2 -2 IR spectrum of residue.

Study No. : 14115
Sample : [1] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : K Oyama

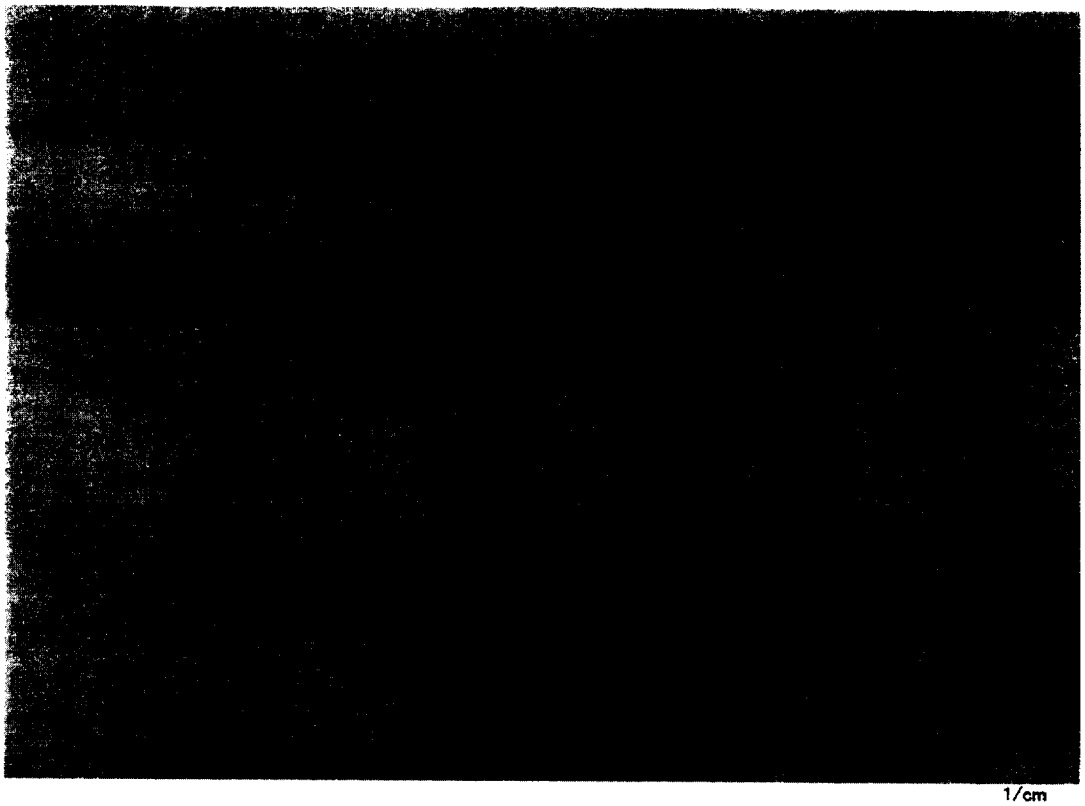


Fig.2 -3 IR spectrum of residue.

Study No. : 14115
Sample : [2] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : K. Oyama

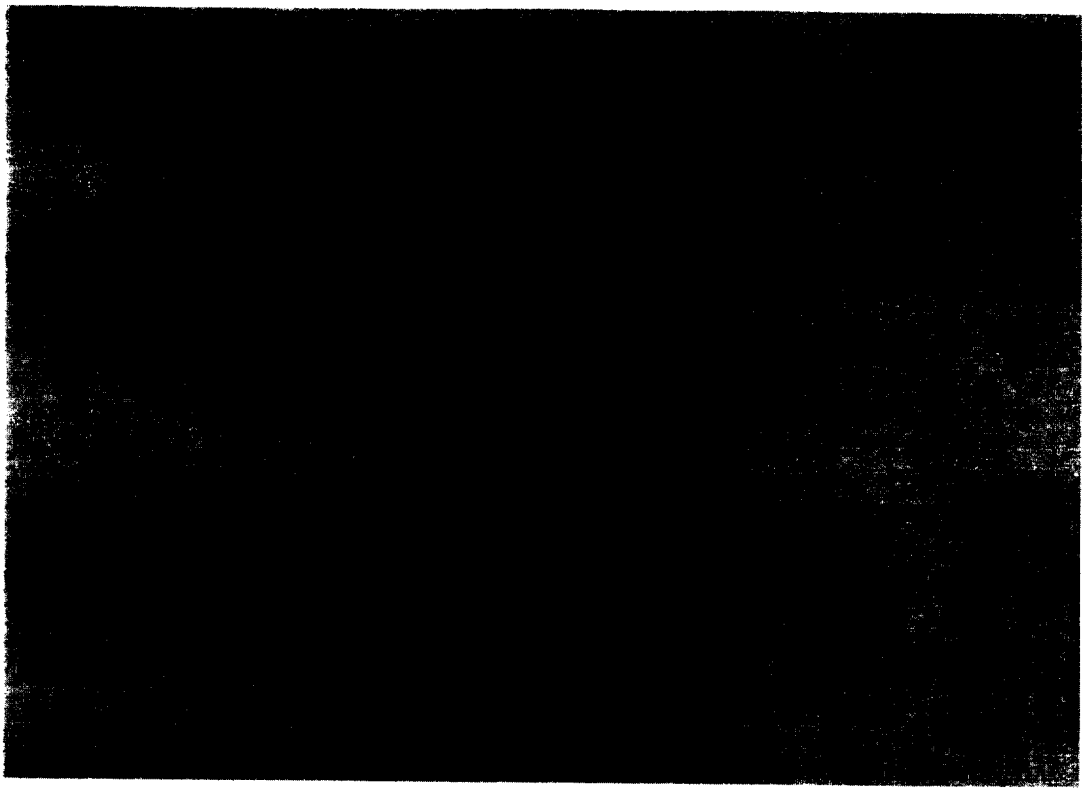


Fig.2 -4 IR spectrum of residue.

Study No. : 14115
Sample : [3] Sludge + test item
Method : KBr tablet
Date : August 26, 2003
Name : k Oyama

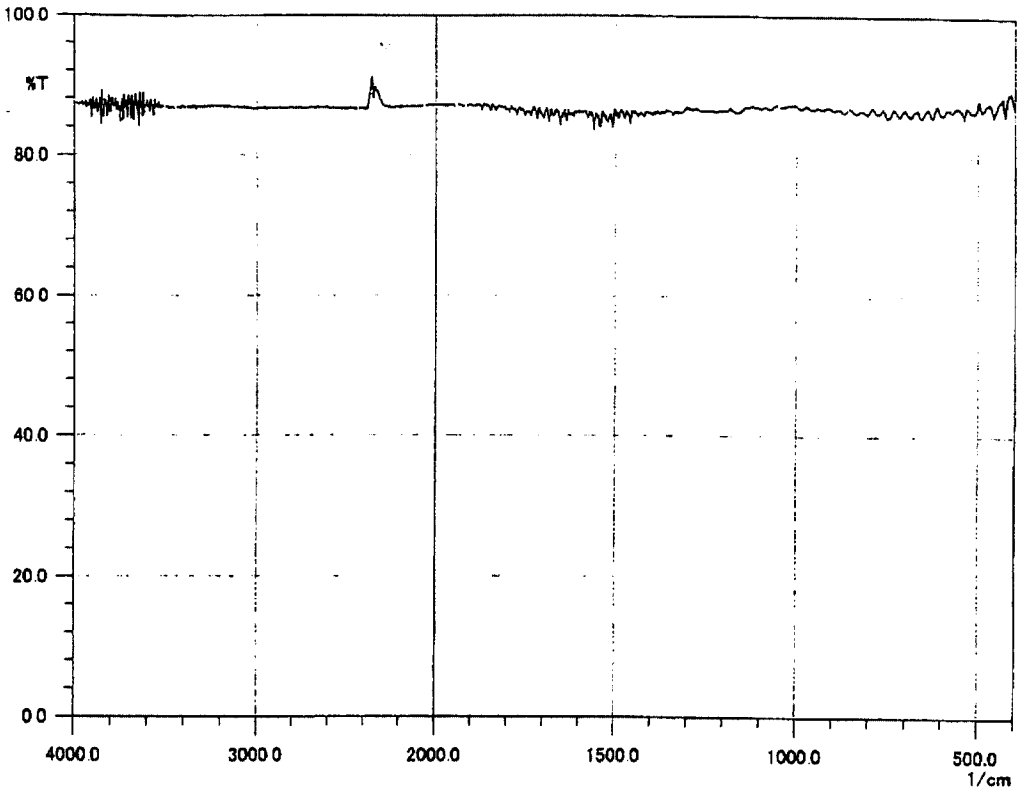


Fig.2 -5 IR spectrum of residue.

Study No. : 14115
Sample : [5] Control blank
Method : KBr tablet
Date : August 26, 2003
Name : k. Oyama

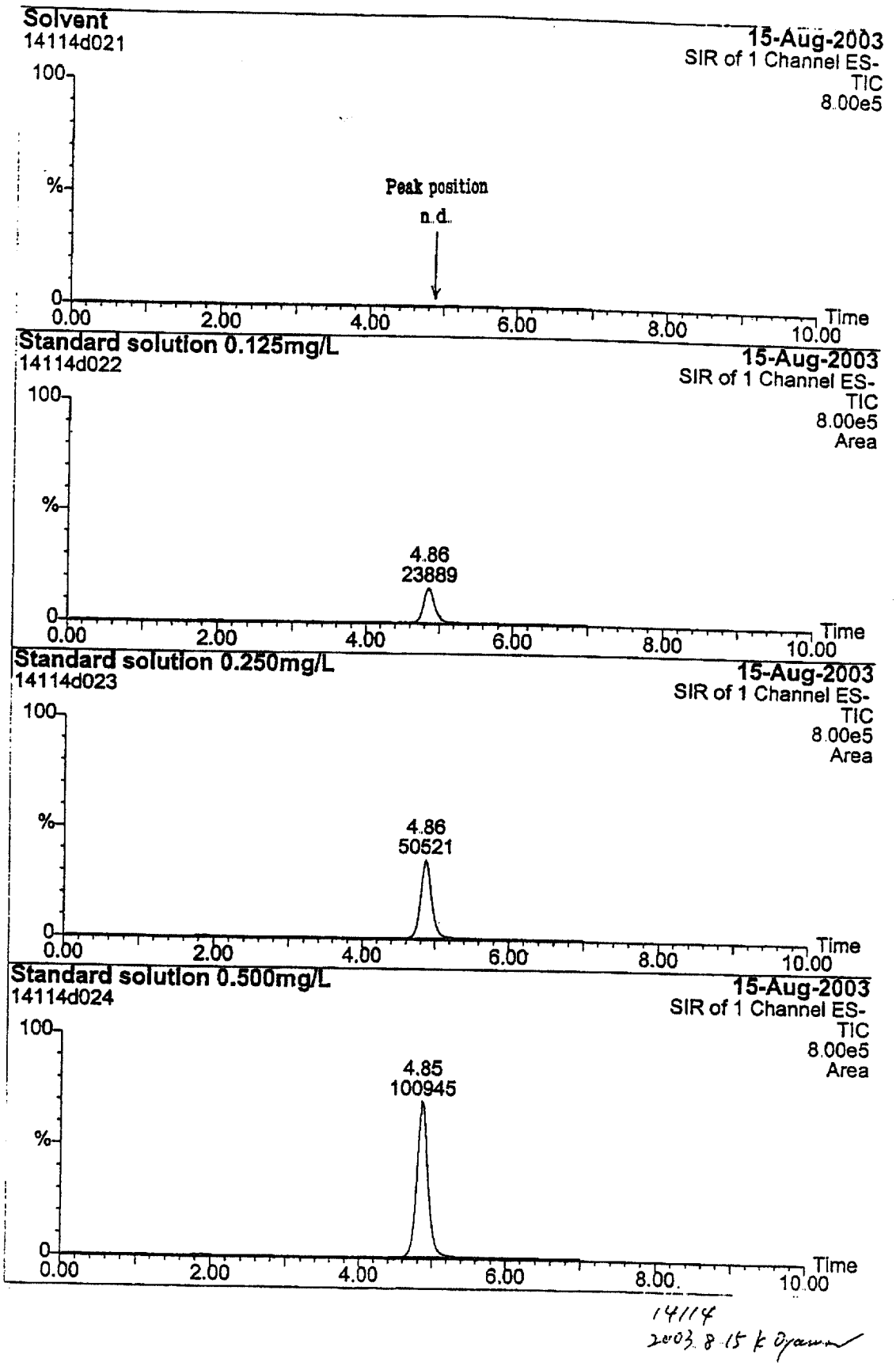
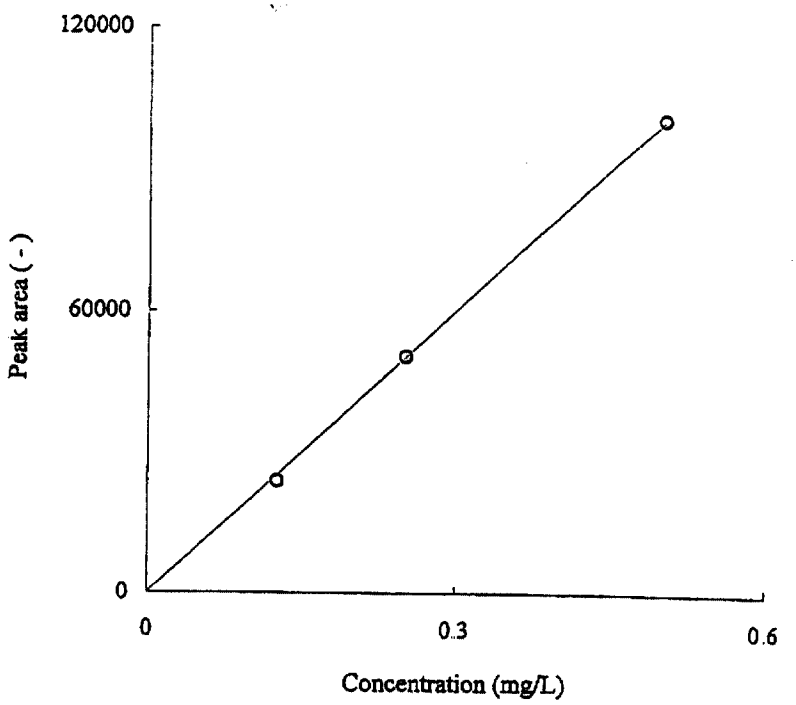


Fig.3 - 1 Mass fragmentogram of LC-MS analysis for calibration curve (perfluorooctanoic acid).

2003. 8 22 7E - K.Oyama
14115

Study No. 14114



y = 201414x
r = 1.00

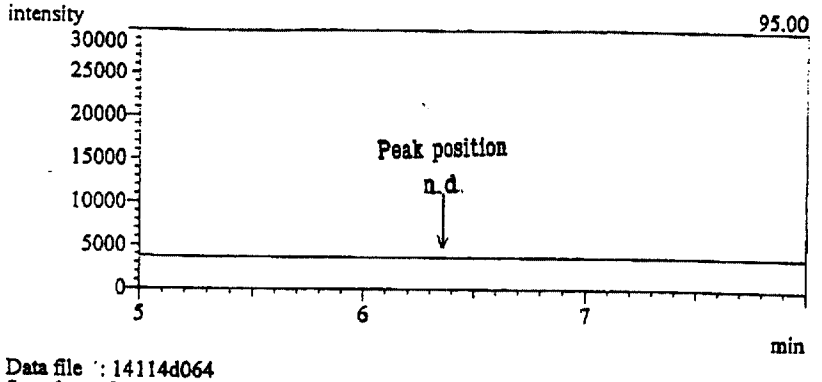
Concentration (mg/L)	Peak area (-)
0.125	23889
0.250	50521
0.500	100945

Fig. 3 - 2 Calibration curve of LC-MS analysis for perfluorooctanoic acid.

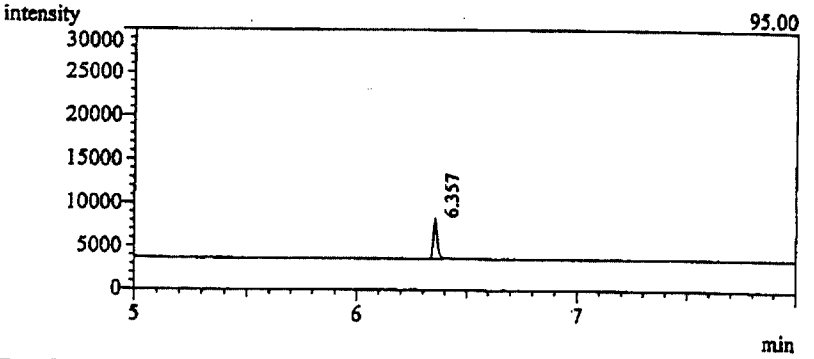
August 22, 2003 Name K. Oyama

2003 8.7 JE- Kiyama
14114-

Data file : 14114d063
Sample : Solvent

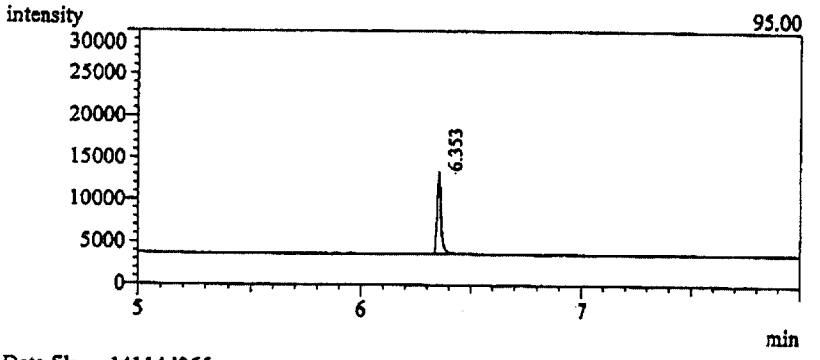


Data file : 14114d064
Sample : Standard solution 0.375mg/L



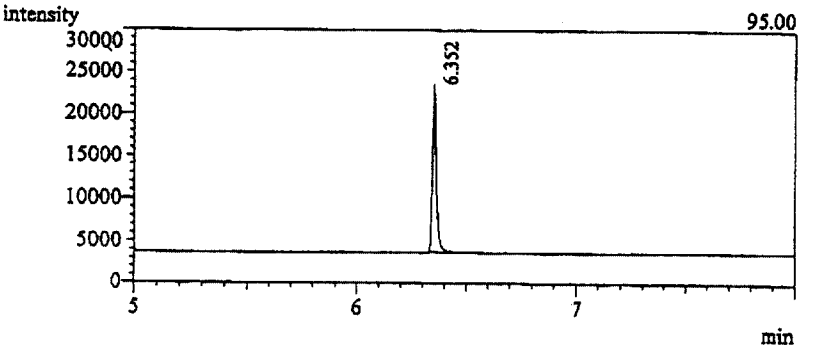
Peak	R.T.	Peak area
1	6.357	6136

Data file : 14114d065
Sample : Standard solution 0.750mg/L



Peak	R.T.	Peak area
1	6.353	12339

Data file : 14114d066
Sample : Standard solution 1.50mg/L



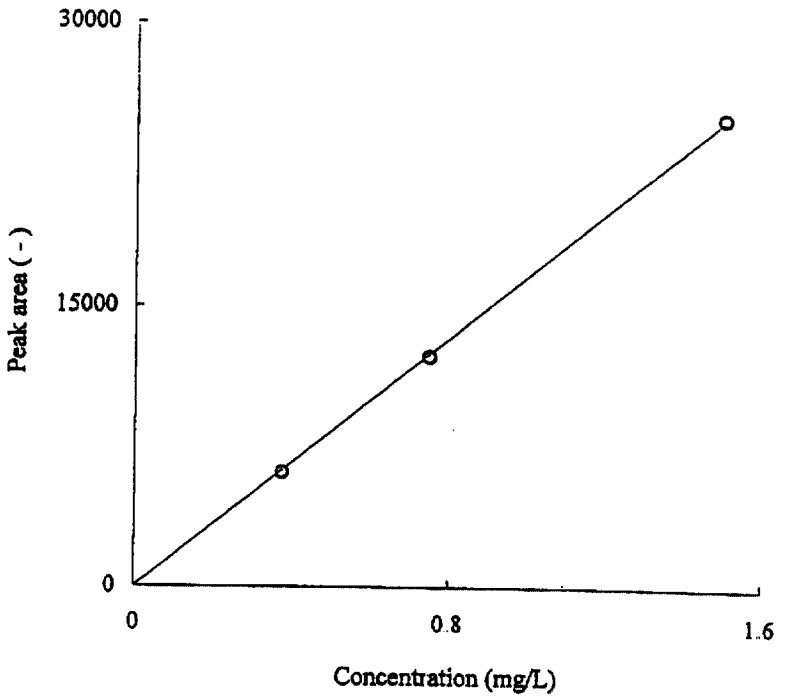
Peak	R.T.	Peak area
1	6.352	25069

Date : August 7, 2003 Name : K Ojama

Fig.4 - 1 Mass fragmentogram of GC-MS analysis for calibration curve (2-(perfluorooctyl)ethanol).

2003 p.11 3E- K.Oyama
14115

Study No. 14114



y = 16646x
r = 1.00

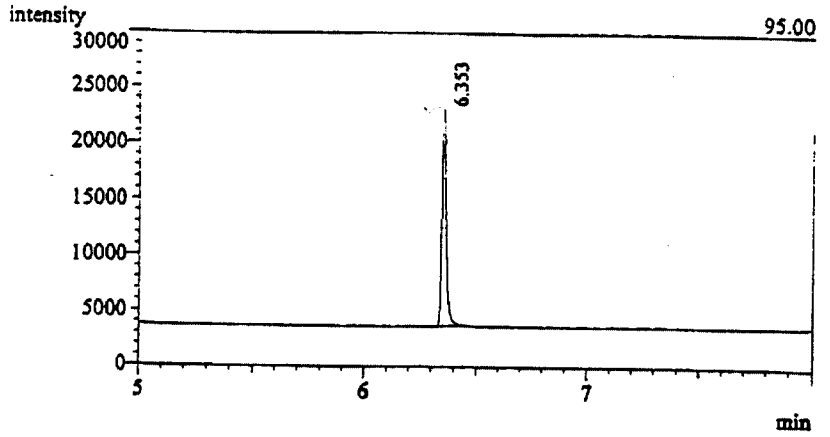
Concentration (mg/L)	Peak area (-)
0.375	6136
0.750	12339
1.50	25069

Fig. 4 - 2 Calibration curve of GC-MS analysis for 2-(perfluorooctyl)ethanol.

August 11, 2003 Name K. Oyama

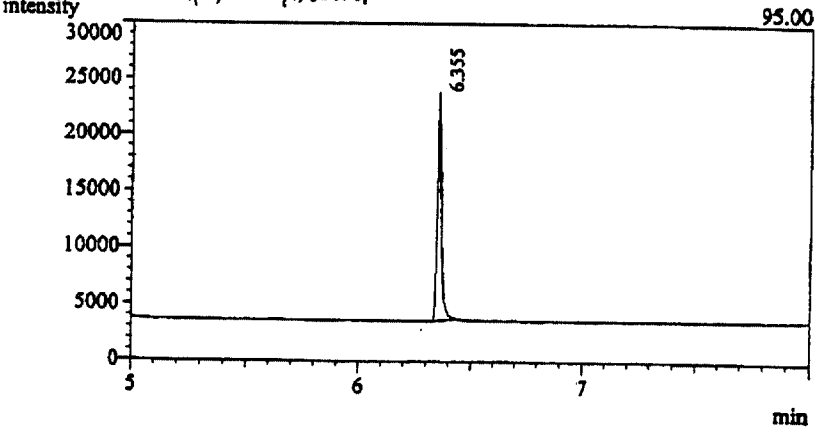
2003. 9. 11 2c- K Ojama
14114

Data file : 14114d067
Sample : Standard solution 1.50mg/L



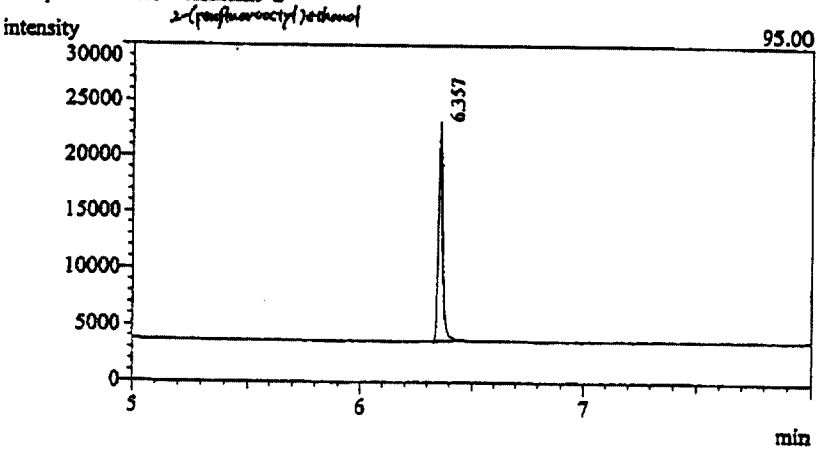
Peak	R.T.	Peak area
1	6.353	26329

Data file : 14114d068
Sample : Water + ~~test-item~~ -1
2-(perfluorooctyl)ethanol



Peak	R.T.	Peak area
1	6.355	26222

Data file : 14114d069
Sample : Water + ~~test-item~~ -2
2-(perfluorooctyl)ethanol

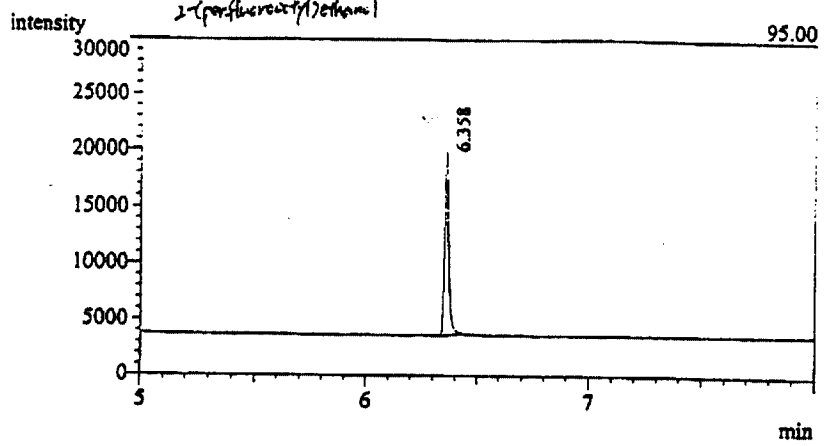


Peak	R.T.	Peak area
1	6.357	25861

Date : August 7, 2003 Name : K. Ojama

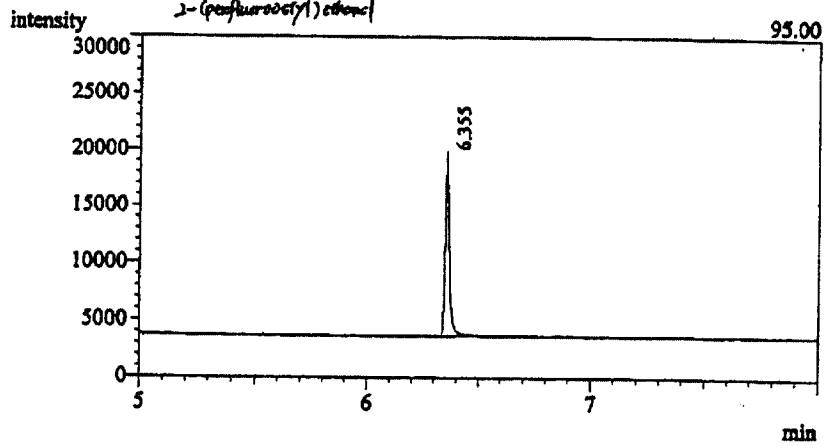
Fig.5 - 1 Mass fragmentogram of GC-MS analysis for recovery and blank test (analysis of 2-(perfluorooctyl)ethanol).

Data file : 14114d070
Sample : Sludge + ~~test-item~~ -1



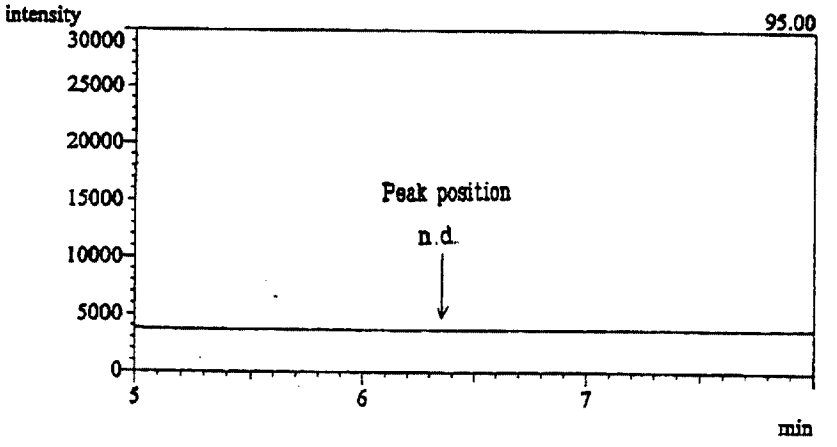
Peak	R.T.	Peak area
1	6.358	21196

Data file : 14114d071
Sample : Sludge + ~~test-item~~ -2



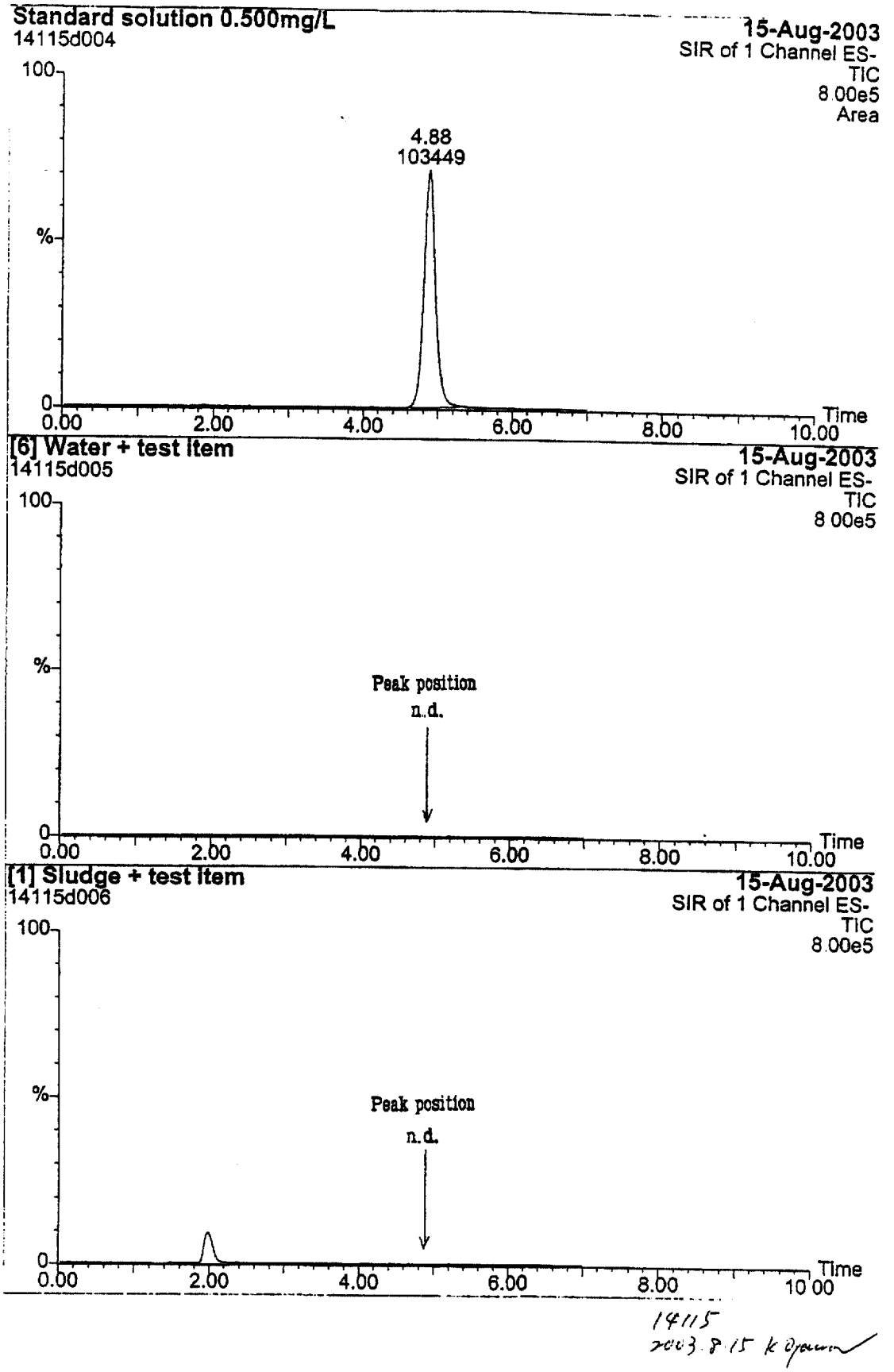
Peak	R.T.	Peak area
1	6.355	21459

Data file : 14114d072
Sample : Control blank



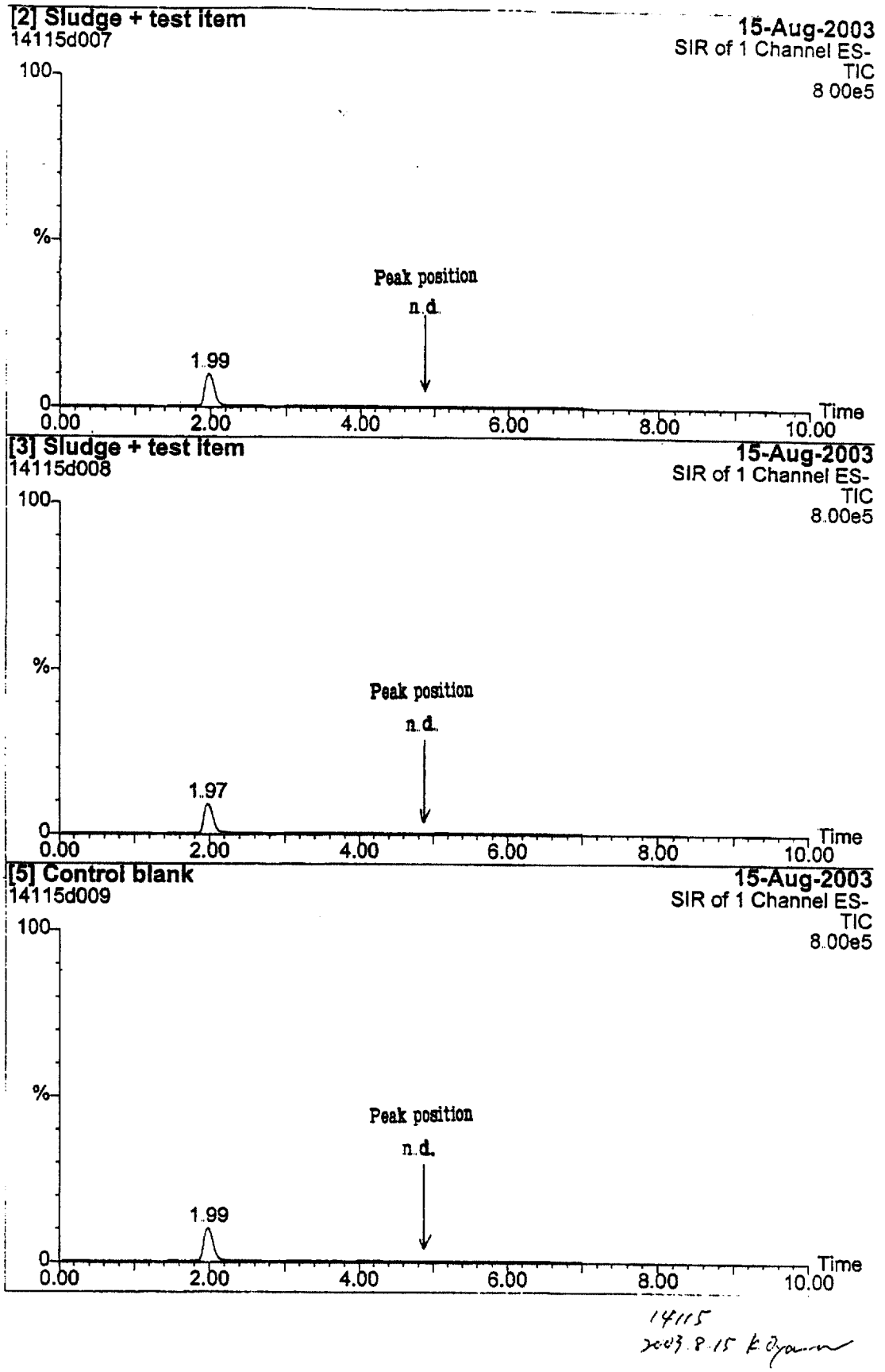
Date : August 7, 2003 Name : K Oyama

Fig.5 - 2 Mass fragmentogram of GC-MS analysis for recovery and blank test (analysis of 2-(perfluorooctyl)ethanol).



14115
2003.8.15 k Ogawa

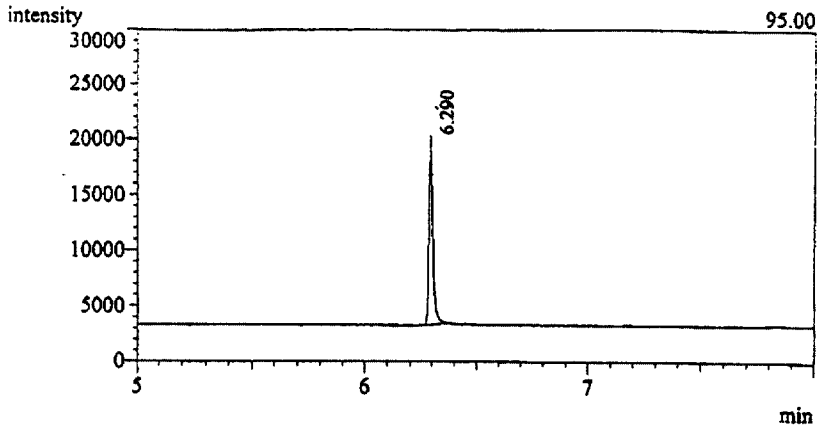
Fig.6 - 1 Mass fragmentogram of LC-MS analysis for test solution (perfluorooctanoic acid).



14115
2003.8.15

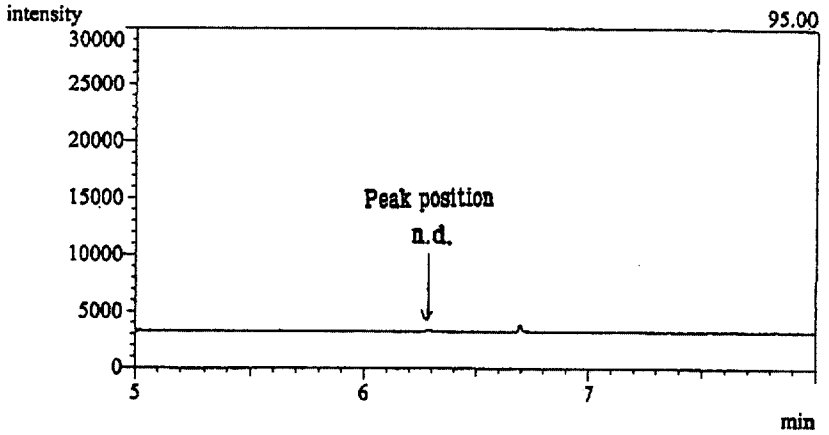
Fig.6 - 2 Mass fragmentogram of LC-MS analysis for test solution (perfluorooctanoic acid).

Data file : 14115d003
Sample : Standard solution 1.50mg/L

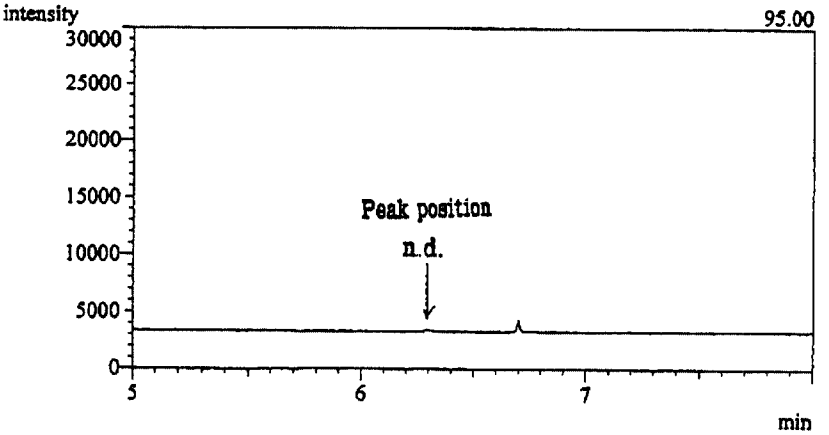


Peak	R.T	Peak area
1	6.290	21672

Data file : 14115d004
Sample : Water + test item



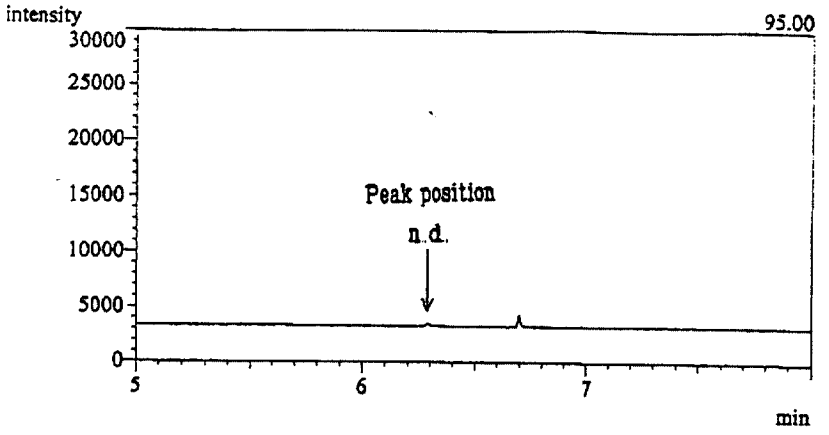
Data file : 14114d005
Sample : Sludge + test item -1



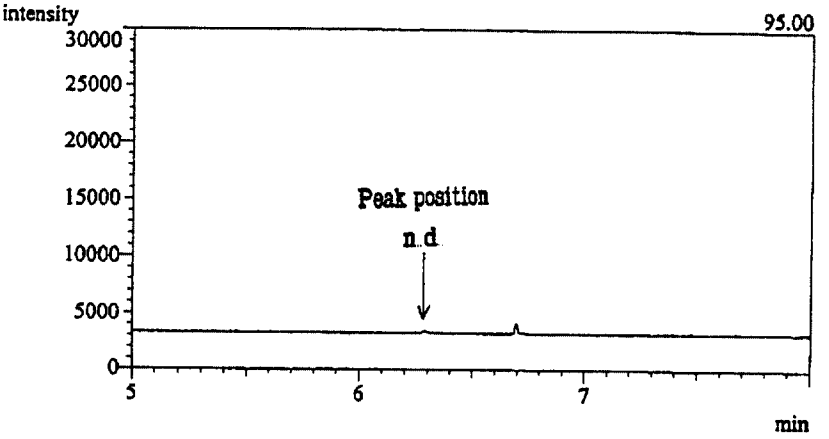
Date : August 18, 2003 Name : k Ogawa

Fig.7 - 1 Mass fragmentogram of GC-MS analysis for test solution (2-(perfluorooctyl)ethanol).

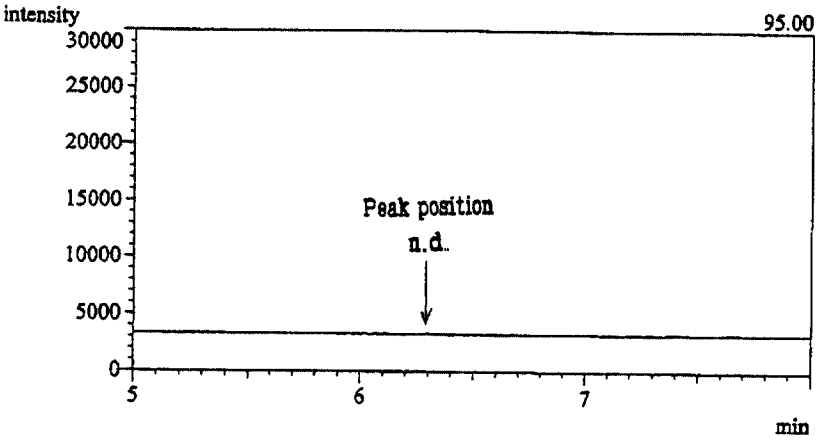
Data file : 14115d006
Sample : Sludge + test item -2



Data file : 14115d007
Sample : Sludge + test item -3



Data file : 14115d008
Sample : Control blank



Date : August 18, 2003 Name : K Oyama

Fig.7 - 2 Mass fragmentogram of GC-MS analysis for test solution (2-(perfluorooctyl)ethanol).

2003.6.17 K.Oyama
14115

Study No. 14114

Instrument MS : Micromass Quattro II, HPLC : Jasco PU-980, AS-950

Sample Perfluorooctanoic acid

HPLC Conditions

Column L-column ODS

Size 15 cm x 4.6 mm I.D.

Eluent Methanol*1/purified water*1 (8/2 V/V) *1 containing 0.1% formic acid

Flow rate 1.0 mL/min

Sample size 20 μL (Solvent Methanol)

MS Conditions

Ionization mode ESI

Detection mode Negative

Function MS

Source conditions (Capillary 3.0 kV, Cone 20 V, Temperature 150 °C)

Mass range (m/z) 100 – 1000

Date June 17, 2003 Operator K. Oyama

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan

Fig.8 - 1 Mass spectrum of perfluorooctanoic acid (analytical condition).

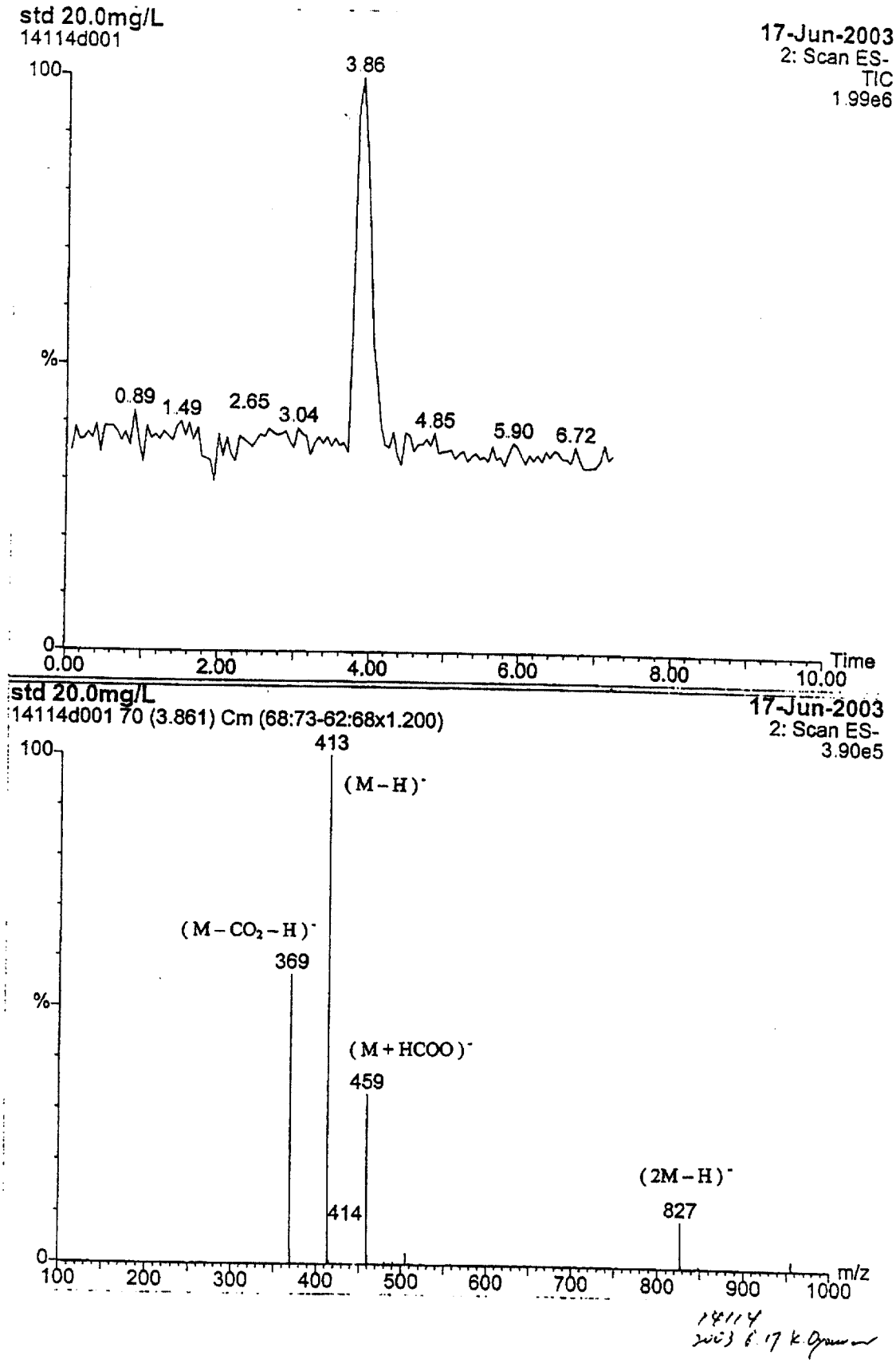


Fig.8 - 2 Mass spectrum of perfluorooctanoic acid (mass spectrum).

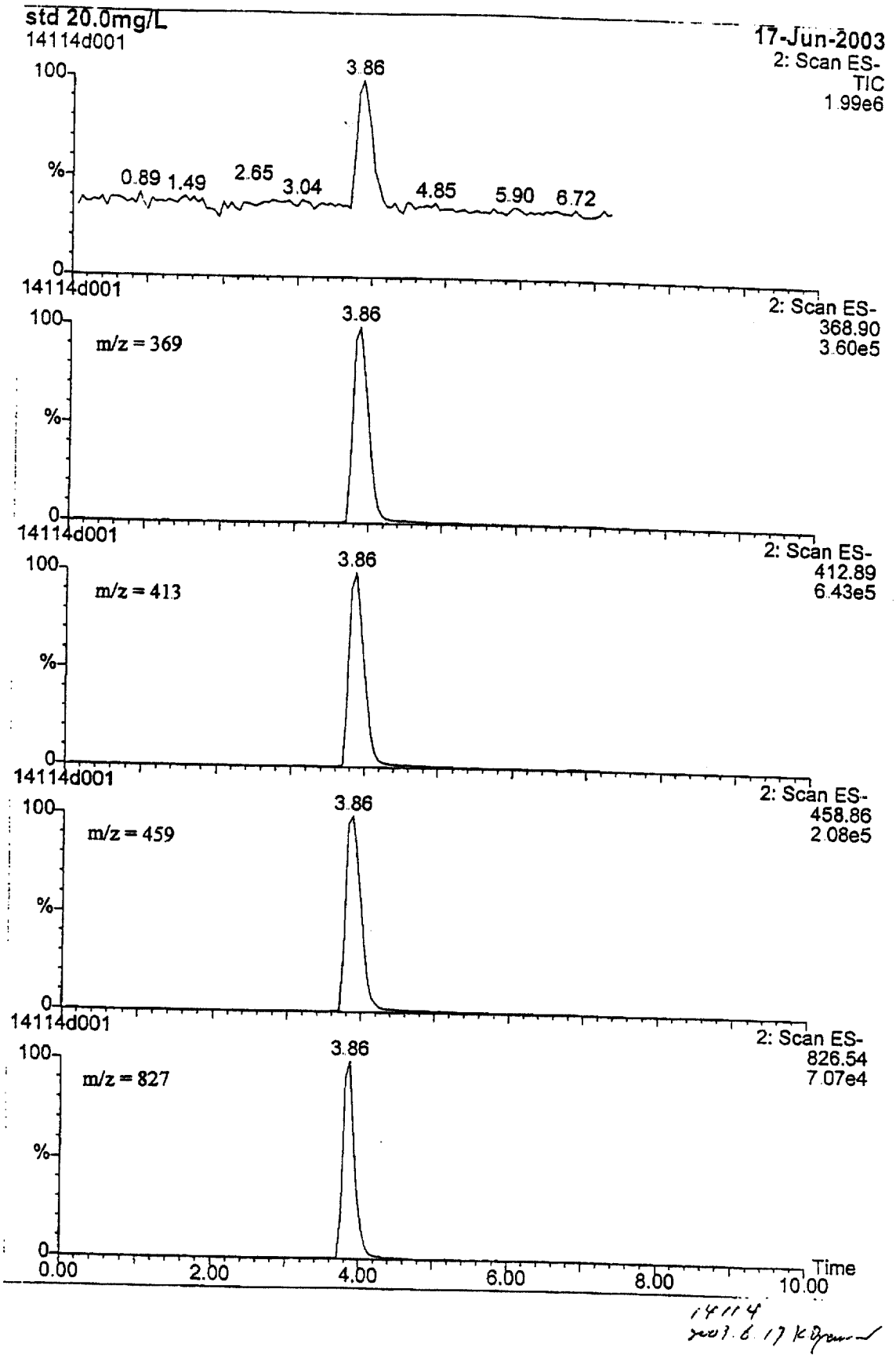


Fig 8 - 3 Mass spectrum of perfluorooctanoic acid (mass chromatogram).

2003 6 11 26- K.Dyama
14115-

Study No. 14114

Instrument Shimadzu QP-5000

Sample 2-(perfluorooctyl)ethanol

GC Conditions

Column HP-FFAP (Fused silica)

Size 25 m x 0.32 mm I.D., Film thickness 0.52 μm

Column temp. 50 °C (3 min) → 170 °C, Temp. rate 30 °C/min

Carrier gas He

Column head pressure 10.0 kPa

Injection temp. 200 °C

Sample size 1 μL (Solvent Methanol)

Inlet mode Splitless

Sampling time 2.0 min

MS Conditions

Ionization Mode EI

Detection Mode Positive

Mass range (m/z) 50 - 600

Interface temp. 230 °C

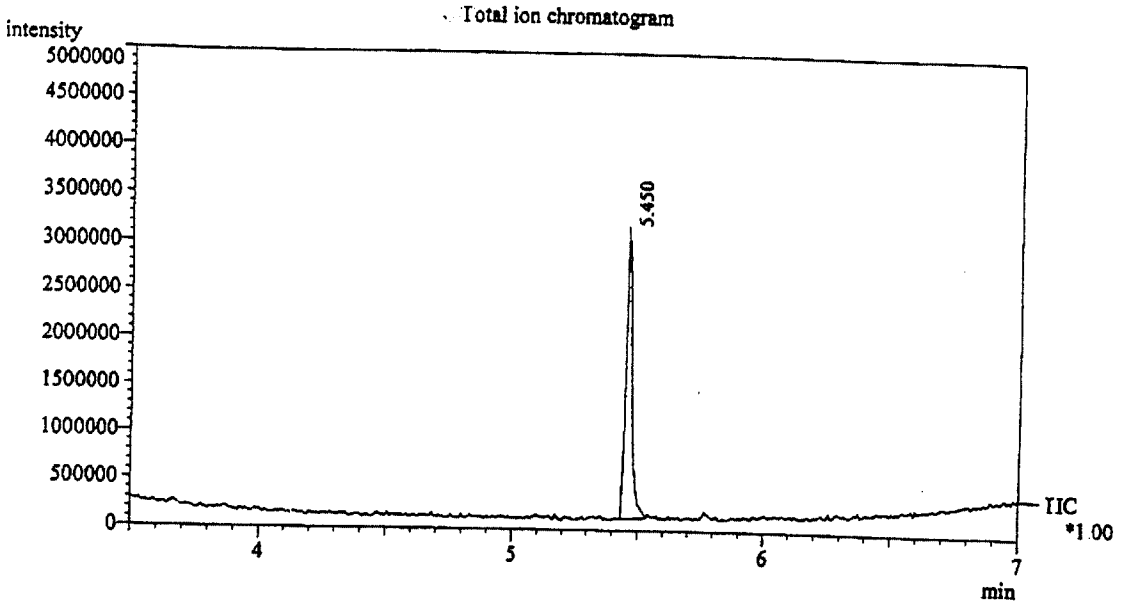
Date June 11, 2003 Operator K. Dyama

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan

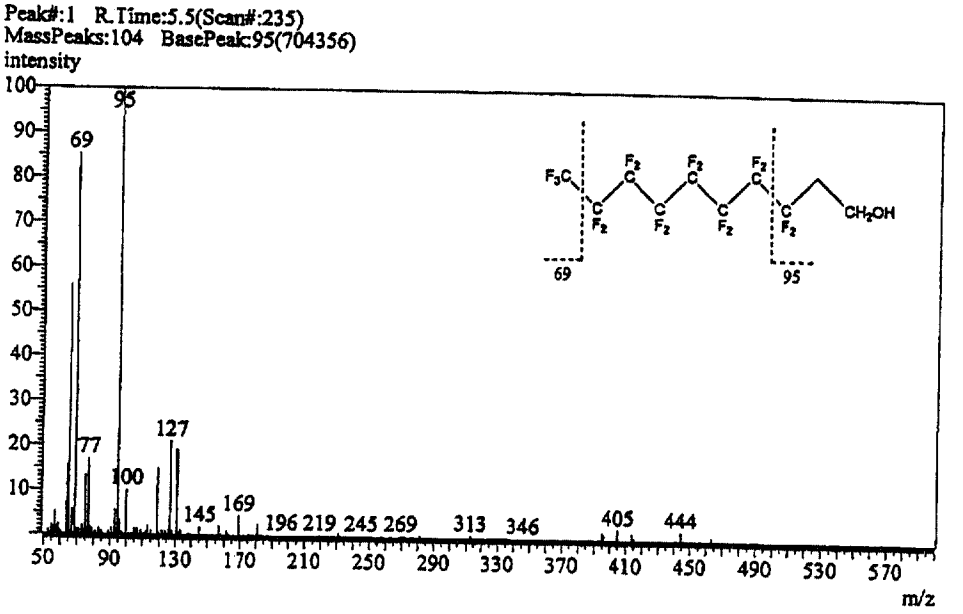
Fig.9 - 1 Mass spectrum of 2-(perfluorooctyl)ethanol (analytical condition).

2003.6.11 JE- K.Ojima
14115

Data file: 14114d001
Sample: standard solution 10.0mg/L



MS spectrum

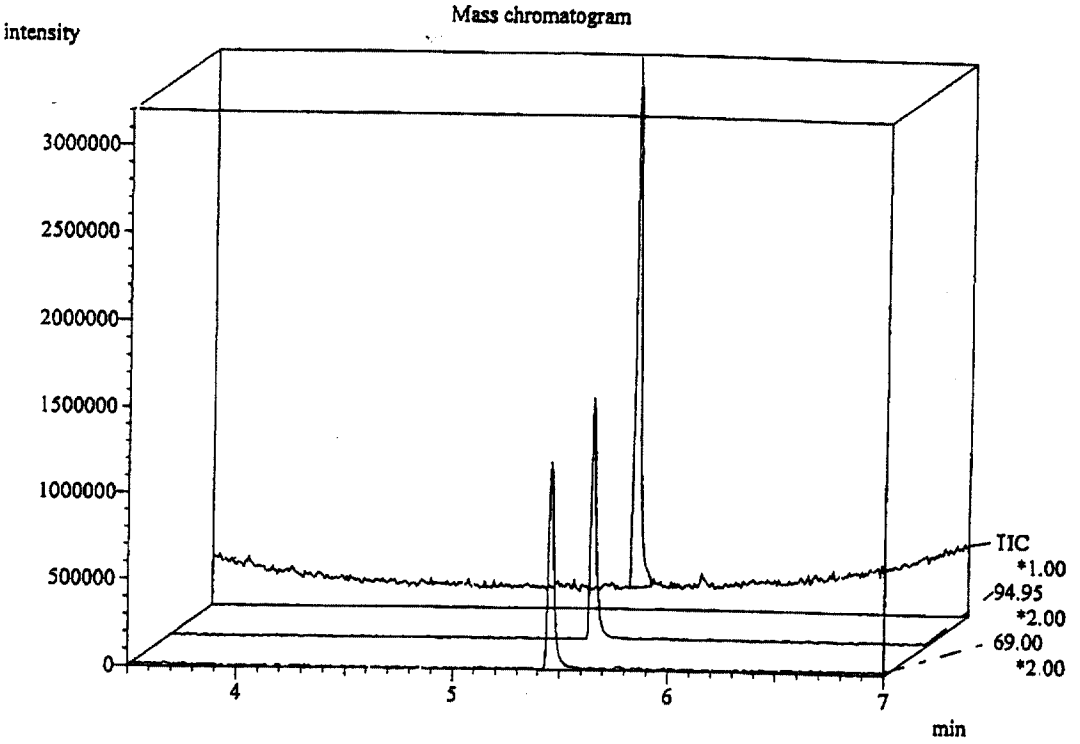


Date : June 11, 2003 Name : K. Ojima

Fig.9 - 2 Mass spectrum of 2-(perfluorooctyl)ethanol (mass spectrum).

2003 6. 11 JC- K Oyama
14115

Data file: 14114d001
Sample: standard solution 10.0mg/L



Date : June 11, 2003 Name : K. Oyama

Fig.9 - 3 Mass spectrum of 2-(perfluorooctyl)ethanol (mass chromatogram).

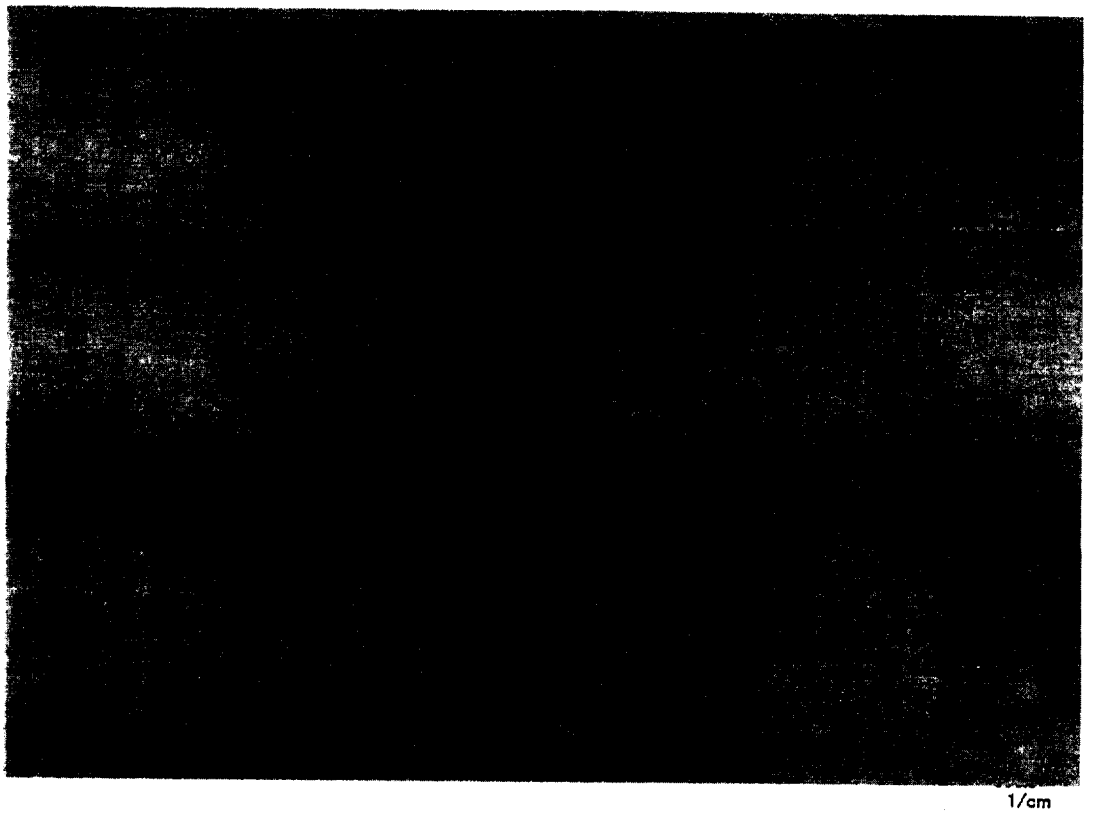


Fig. 10 -1 IR spectrum of test item measured before experimental start.

Study No. : 14115
Sample : Test item
Method : KBr tablet
Date : July 10, 2003
Name : K Oyama

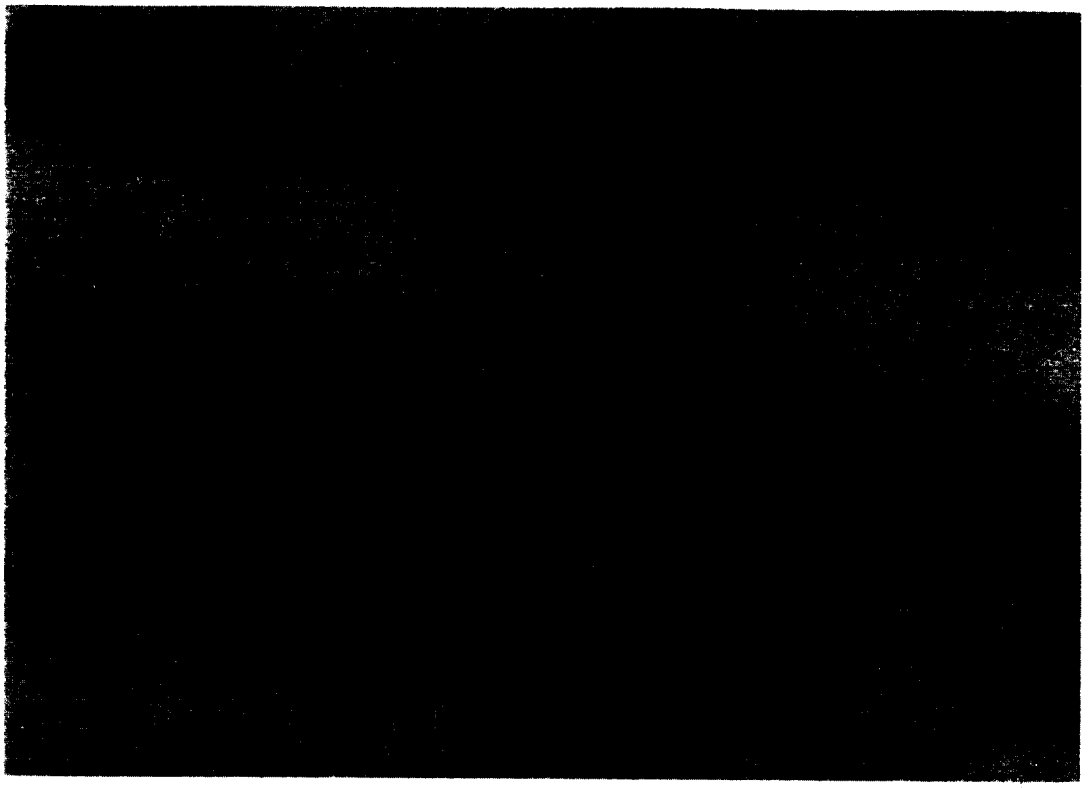
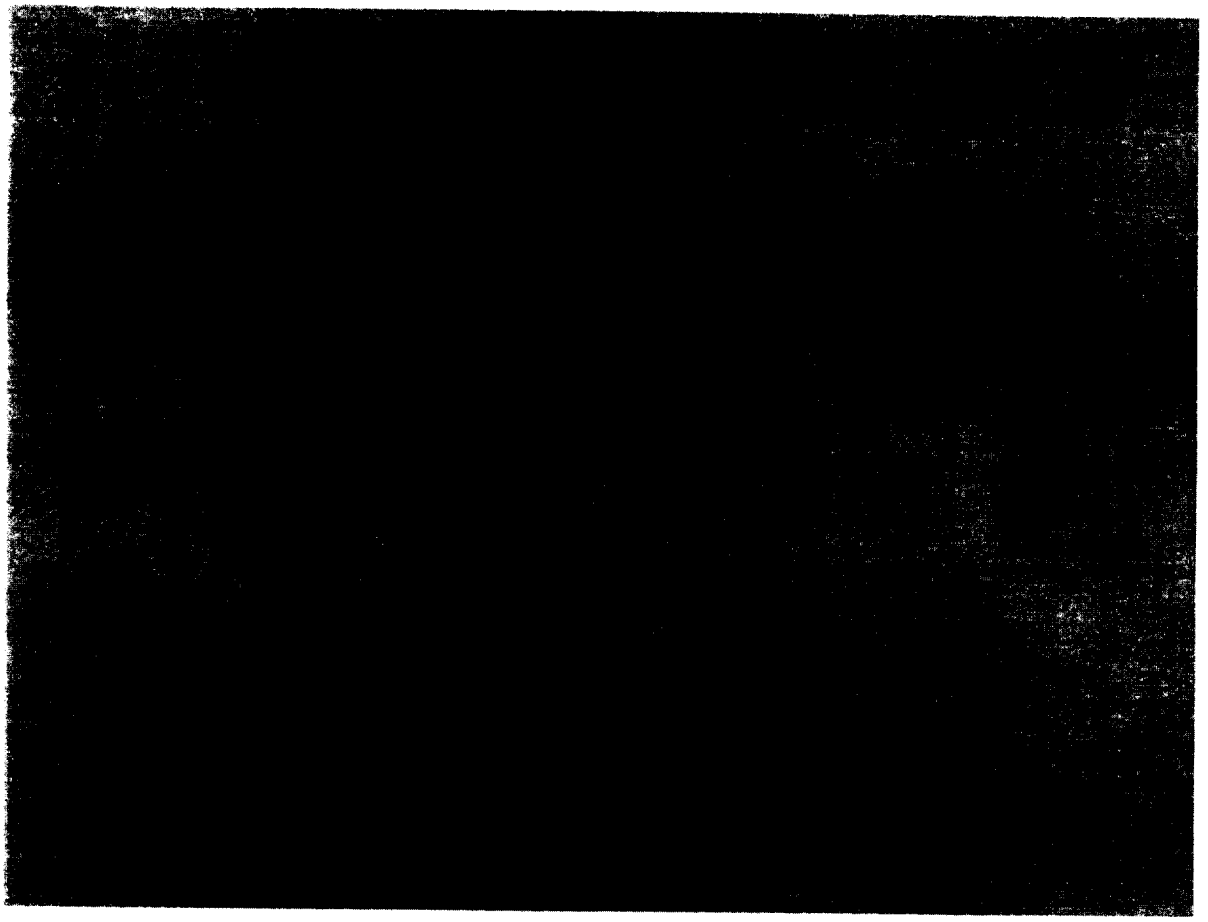


Fig.10-2 IR spectrum of test item measured after experimental completion.

Study No. : 14115
Sample : Test item
Method : KBr tablet
Date : August 27, 2003
Name : k Oyama



Reference IR spectrum supplied by sponsor

Amendment to Final Report

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

1. Title — Biodegradation study of (CBI) by microorganisms

2. Study number — 14115

3. Content — 4.5.3 Recovery test and blank test (page 19)

4. Reason — Writing error

5. Content of correction

" Recovery rate of the test solutions (water + test item)
99.6 %, 98.2 % average 98.9 %
Recovery rate of the test solutions (sludge + test item)
80.5 %, 81.5 % average 81.0 % "

is corrected to

" Recovery rate of the test solutions (water + 2-(perfluorooctyl)ethanol)
99.6 %, 98.2 % average 98.9 %
Recovery rate of the test solutions (sludge + 2-(perfluorooctyl)ethanol)
80.5 %, 81.5 % average 81.0 % ".

6. Approval

Date October 10, 2003

Study Director Signed in original

Yasuko Matsunobu