CERI

Receipt number	682-16-E-7724
Study number	97724

FINAL REPORT

Activated sludge respiration inhibition test of APFHx (C-1500N)

June, 2017

Chemicals Evaluation and Research Institute, Japan, Kurume

GLP STATEMENT

Chemicals Evaluation and
Research Institute, Japan, Kurume

Sponsor DAIKIN INDUSTRIES, LTD.

Title Activated sludge respiration inhibition test of APFHx (C-1500N)

Study number 97724

The study described in this report was conducted in compliance with the following GLP principles:
OECD Principles of Good Laboratory Practice, November 26, 1997, ENV/MC/CHEM (98)17

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

Date June 1, 2017

Study Director

QUALITY ASSURANCE STATEMENT

Chemicals Evaluation and Research Institute, Japan, Kurume

Sponsor: DAIKIN INDUSTRIES, LTD.

Title: Activated sludge respiration inhibition test of APFHx (C-1500N)

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I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

Item of inspection	Date of inspection	Date of report
Study plan	March 17, 2017	March 17, 2017
Test conduct	March 27, 2017 March 28, 2017	March 28, 2017
Raw data and draft final report	May 23, 2017	May 23, 2017
Final report	June 1, 2017	June 1, 2017

Date June 1, 2017

Personnel of Quality Assurance Unit:

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1. Title

   Activated sludge respiration inhibition test of APFHx (C-1500N)

2. Sponsor

   Name DAIKIN INDUSTRIES, LTD.

   Address 1-1 Nishi Hitotsuya, Settsu-shi, Osaka 566-8585, Japan

3. Test facility

   Name Chemicals Evaluation and Research Institute, Japan, Kurume (CERI Kurume)

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

4. Objective

   The objective of this study is to determine the 3-hour median effective concentration (EC_{50}) and no observed effect concentration (NOEC) by conducting an activated sludge respiration inhibition test with APFHx (C-1500N).

5. Test method

   OECD Guidelines for the Testing of Chemicals, No.209, July 22, 2010, "Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)"

6. GLP principle

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

7. Dates

Study initiation date	March 16, 2017
Experimental starting date	March 28, 2017
Experimental completion date	March 28, 2017
Study completion date	June 1, 2017

8. Storage of test item, raw data, etc.

The study plan (original), the final report (original), the raw data, documents concerning the study presented by the sponsor and other reports are stored in the archives of this laboratory. The test item will be returned to the sponsor. The storage period is 10 years after the study completion date. Treatment of the raw data, etc. after the storage period (continue, reject, or return) is discussed with the sponsor.

9. Personnel

Study Director

Study personnel (Operation of test)

10. Approval of final report

Date June 1, 2017

Study Director

11. Summary

Test item

APFHx (C-1500N)

Objective

The objective of this study is to determine the 3-hour median effective concentration (EC_{50}) and no observed effect concentration (NOEC) by conducting an activated sludge respiration inhibition test with APFHx (C-1500N).

Test method

OECD Guidelines for the Testing of Chemicals, No.209, July 22, 2010, "Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)"

Conditions of incubation

Inoculum	Activated sludge obtained from an aeration reactor of a sewage treatment plant receiving predominantly domestic sewage
Concentration of test item	1000 mg/L as nominal loading concentration
Replicate	3 replicates/exposure level 6 replicates/control
Volume of test solution	250 mL
Aeration rate	0.5–1 L/min
Exposure duration	3 hours
Test temperature	20 ± 2°C

Measurement item

Measurement of dissolved oxygen concentration to calculate oxygen consumption rate

Results

a) Oxygen consumption rate and percentage inhibition of respiration after 3 hours of exposure

Concentration (mg/L)	Oxygen consumption rate (mg O_2/L/h)	Percentage inhibition of respiration (%)	Average percentage inhibition (%)
1000	46.3, 47.9, 48.0	0, -3, -4	-2

Oxygen consumption rate in blank control: average 46.3 mg O_2/L/h

(at the beginning: 47.8, 49.2 and 47.7 mg O_2/L/h, at the end: 45.9, 44.4 and 42.9 mg O_2/L/h)

b) EC_{50} and NOEC

EC_{50} (3 h) > 1000 mg/L

NOEC (3 h) ≥ 1000 mg/L

12. Test materials

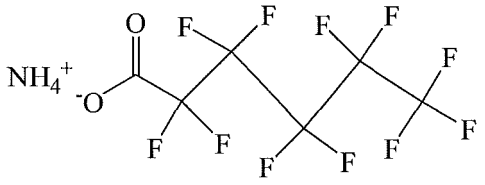
12.1 Test item

a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-Undecafluorohexanoic acid, ammonium salt
Another name	APFHx (C-1500N)
CAS Number	21615-47-4

b) Chemical structure etc.

Structural formula



Molecular formula	$C_6H_4F_{11}NO_2$
Molecular weight	331.08

c) Test sample

Purity of test item	50%
Impurity	Water 50%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150E62004

The test sample was treated by correction with the purity of test item.

d) Physicochemical properties

Appearance	Clear and colorless liquid

e) Storage conditions

The test sample was stored in a dark storage place at room temperature.

f) Identification and stability of test item

The infrared (IR) spectrum of the test item measured at this laboratory was confirmed to be identical to that provided by the sponsor (see Fig. 2 and Reference 1).

The stability of the test item was confirmed by comparing the IR spectrum of the test item after the completion of the experiment with that before the start of the experiment (see Fig. 2).

g) Safety and handling

In order to avoid inhalation and contact with the skin and eyes, chemically resistant gloves, mask, safety glasses, and white coats were worn when handling test item.

12.2 Reference item

a) Chemical name etc.

Chemical name	3,5-Dichlorophenol
CAS number	591-35-5

b) Supplier, lot number etc.

Purity	100.0%
Supplier	Wako Pure Chemical Industries
Lot number	LKH3471

12.3 Activated sludge

Inoculum source	Activated sludge obtained from an aeration reactor of a sewage treatment plant receiving predominantly domestic sewage
Sampling site	Kurume central sewage treatment center (Kurume-shi, Fukuoka, Japan)
Sampling date	March 27, 2017
Concentration of suspended solid	2910 mg/L
Method:	In accordance with Japanese Industrial Standards (JIS) K 0102:2016 Section 14.1, 20 mL of the activated sludge was filtrated, and the residue was dried for 2 hours at 110°C and weighted.
Date:	March 28, 2017

The activated sludge (5 L) was fed with the synthetic sewage (250 mL, see Section 13.2 a)) and cultivated for 21.5 hours at 20 ± 2°C under aerobic condition in order to acclimate the test temperature. The activated sludge was then used. Acclimation to the test item was not performed.

13. Performance of respiration inhibition test

13.1 Test vessels and apparatus

Test vessel	Approximately 300 mL glass vessel
Aerator	Air pump (controllable flow rate at 0.5–1 L/min.)
Dissolved oxygen analyzer	B-103Z (Iijima Electronics)
Measurement bottle	102 mL glass incubator bottle

13.2 Preparations for test

a) Preparation of synthetic sewage

500 mL of the synthetic sewage was prepared at the same proportion as the following method; the following reagents were dissolved in 1 L of purified water. The pH of the prepared synthetic sewage was 7.2. The pH was not adjusted because the pH was within 7.5 ± 0.5. The synthetic sewage was prepared the day before the test and was stored in a refrigerator until use.

Peptone	16 g
Meat extract	11 g
Urea	3 g
Sodium chloride	0.7 g
Calcium chloride dihydrate	0.4 g
Magnesium sulfate heptahydrate	0.2 g
Dipotassium hydrogen phosphate	2.8 g

b) Preparation of stock solutions

1) Stock solution of test item

The test sample (2000 mg, i.e., 1000 mg as the test item) was accurately weighed and was filled up to 100 mL with purified water. 10000 mg/L solution of the test item was then obtained.

2) Stock solution of 3,5-dichlorophenol

3,5-Dichlorophenol (100 mg) was accurately weighed and was dissolved in approximately 80 mL of purified water. The pH of this solution was adjusted to 7.5 with 1 mol/L sodium hydroxide solution. This solution was filled up to 100 mL with purified water, and 1000 mg/L stock solution of 3,5-dichlorophenol was obtained.

13.3 Preparation of test solutions

The test solutions were prepared by the following procedure, and pH of all the test solutions was measured. The pH of the test solutions (reference mixture and blank control used at the beginning of the test (n=3)) was adjusted to 7.5 with 1 mol/L sodium hydroxide solution because the pH was below 7.0. The pH of the other test solutions was between 7.0 and 7.1 and was not adjusted.

a) Test mixture

The 10000 mg/L stock solution of the test item (25 mL), which was prepared in Section 13.2 b) 1), the synthetic sewage (8 mL), the purified water (92 mL) and the activated sludge (125 mL) were added to each test vessel.

b) Reference mixture

The 1000 mg/L stock solution of 3,5-dichlorophenol (1, 2, 4 and 8 mL), which was prepared in Section 13.2 b) 2), the synthetic sewage (8 mL), the purified water (the volume subtracting the volume of the stock solution of 3,5-dichlorophenol from 117 mL) and the activated sludge (125 mL) were added to each test vessel.

c) Blank control

The synthetic sewage (8 mL), the purified water (117 mL) and the activated sludge (125 mL) were added to each test vessel.

13.4 Conditions of test

In the preliminary test, three concentrations of the test item at 10, 100 and 1000 mg/L were prepared, and their percentages of respiration inhibition were 1%, 11% and 12%, respectively, after 3 hours. The respiration inhibition at 10 mg/L was not observed. Although the test item was soluble in water, the concentration dependence of toxicity was not observed between 100 mg/L and 1000 mg/L. The results suggested that the toxicity of the test item to microorganisms was not observed in the test mixture up to 1000 mg/L. Therefore, the test mixture for the definitive test was prepared as limit test with a concentration of 1000 mg/L as nominal loading concentration.

Test concentration	Test mixture	1000 mg/L (nominal loading concentration)
	Reference mixture	4.0, 8.0, 16 and 32 mg/L (geometric series with a factor of 2)
Replicate	Test mixture	3 replicates/exposure level
	Reference mixture	1 replicate/exposure level
	Blank control	6 replicates (each 3 replicates at the beginning and the end of the exposure period in the test)
Volume of test solution	250 mL	
Aeration rate	0.5–1 L/min	
Exposure duration	3 hours	
Test temperature	20 ± 2°C	
Room	Experimental room 3B	

13.5 Measurement of the dissolved oxygen concentration

After the 3 hours of exposure, the measurement bottle was filled with the test solution promptly and softly with attention to avoid foaming. Afterwards, the concentration of dissolved oxygen in the test solution was measured continuously for 10 minutes with stirring.

13.6 Estimation of EC_{50} and NOEC

a) Calculation of percentage inhibition of respiration

The concentration of dissolved oxygen (mg O_2/L) was plotted against the measuring time (h) for each test solution, and a regression formula was derived from the linear part in the graph. The oxygen consumption rate (mg O_2/L/h) was obtained from the absolute value of slope of the regression formula.

The percentage inhibition of respiration was then calculated by the following equation:

$$\text{Percentage inhibition of respiration (\%)} = (1 - \frac{RS}{RC}) \times 100$$

RS : Oxygen consumption rate in test or reference mixture (mg O_2/L/h)

RC : Average oxygen consumption rate in blank control (mg O_2/L/h)

b) Estimation of EC_{50}

The EC_{50} of the test item was estimated as " >1000 mg/L" because less than 50% of inhibition rate was obtained within the exposure level (see Section 15.1 a)).

In the reference mixture, the percentage inhibition in each exposure levels was plotted on semi-logarithmic graph against the corresponding concentration. The EC_{50} of 3,5-dichlorophenol was calculated from the regression formula obtained by probit analysis (see Fig.1). The EC_{50} was determined using computer program (running on Microsoft software "Excel") constructed by our laboratory.

c) Estimation of NOEC

The NOEC of the test item was estimated by the following statistical analyses. Regarding the oxygen consumption rate, after *F* test was done to determine the homogeneity of variance for the data, Aspin-Welch *t*-test was used to estimate the significant difference in comparison with the control. The statistical analysis was conducted using computer program (running on Microsoft software "Excel") constructed by our laboratory. NOEC was determined by the results of statistical analysis and was estimated as " ≥the highest test concentration" since respiration inhibition was not observed in all exposure levels.

13.7 Validity of test

a) The EC_{50} of 3,5-dichlorophenol as reference item was 11 mg/L [See Section 15.1 b) and Fig. 1], and this value met the criterion (2–25 mg/L).

b) The coefficient of variation of the oxygen consumption rate in blank controls between at the beginning and at the end of the exposure period was 5.9%, and this value met the criterion (less than 30%).

c) The oxygen consumption rate per gram (dry weight) of activated sludge per hour (see the following equation) in blank control was average 31.8 mg O_2/g/h, and this value met the criterion (≥ 20 mg O_2/g/h).

Oxygen consumption rate of per gram (dry weight) of activated sludge per hour (mg O_2/g/h)

$$= \frac{RC_X \times V}{SS \times V_S} \times 1000$$

RC_X : Average oxygen consumption rate in blank control (mg O_2/L/h)

V : Volume of test solution (L)

SS : Concentration of suspended solid in activated sludge (mg/L)

V_S : Additive amount of activated sludge (L)

13.8 Treatment of numerical values

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

14. Factors that affected reliability of test

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

15. Results and discussion

15.1 Results

a) Oxygen consumption rate and percentage inhibition of respiration

Table 1 Results of oxygen consumption rate and percentage inhibition of respiration after 3 hours of exposure

Test solution	Nominal loading concentration (mg/L)	Oxygen consumption rate (mg O_2/L/h)	Percentage inhibition of respiration (%)	Average percentage inhibition (%)
Test mixture	1000	46.3, 47.9, 48.0	0, -3, -4	-2
Reference mixture	4.0	39.4	15	-
	8.0	29.3	37	-
	16	13.5	71	-
	32	6.76	85	-
Blank control	-	At the beginning: 47.8, 49.2, 47.7 At the end: 45.9, 44.4, 42.9	-	-

Average oxygen consumption rate in blank control: 46.3 mg O_2/L/h

b) EC_{50} and NOEC

Test item EC_{50} (3 h) > 1000 mg/L

NOEC (3 h) ≥ 1000 mg/L (see Table 1)

Reference item EC_{50} (3 h) = 11 mg/L (see Fig. 1)

Table 2 Result of statistical analysis

Test solution	Nominal loading concentration (mg/L)	Statistical analysis	Statistical procedure
Test mixture	1000	n.s.	*F* test Aspin-Welch *t*-test

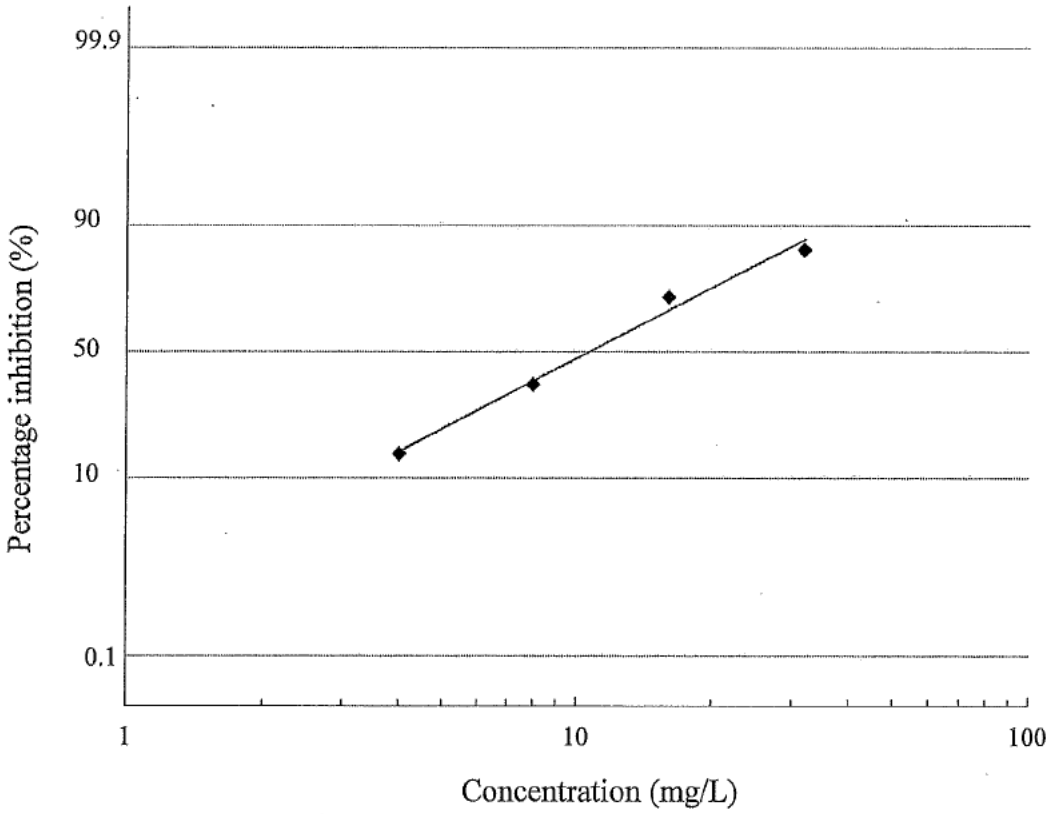
n.s. : no significant difference

15.2 Discussion

The activated sludge was obtained from the aeration reactor of the sewage treatment plant receiving predominantly domestic sewage. This test was valid because all of the validity criteria were met (see Section 13.7).

The average percentage inhibition of respiration of the test mixtures at 1000 mg/L was -2%. Therefore, the EC_{50} of the test item was estimated as " >1000 mg/L". The NOEC estimated using the statistical analyses was ≥ 1000 mg/L in the test because inhibition was not observed within the exposure level.

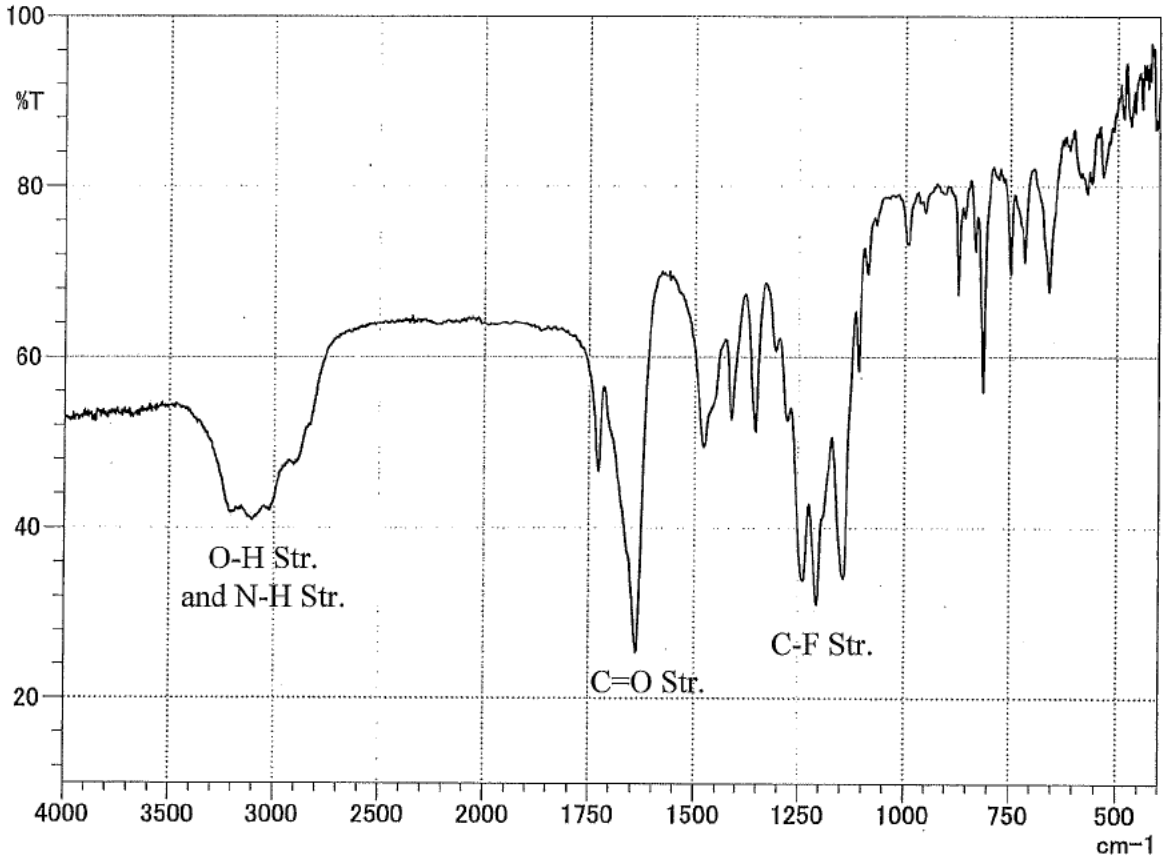
3-hour EC_{50}= 11 mg/L (Probit)



Concentration (mg/L)	Percentage inhibition (%) 3 hours
4.0	15
8.0	37
16	71
32	85

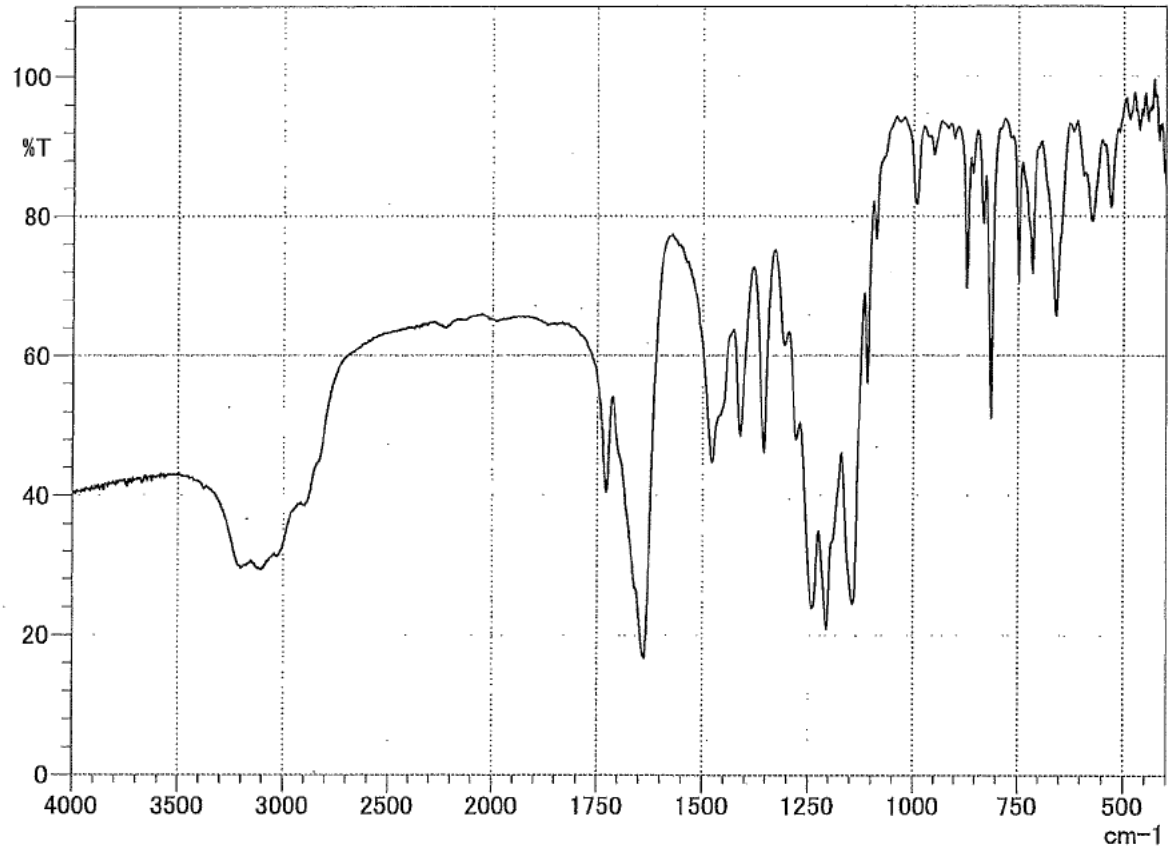
Fig. 1 Concentration - respiration inhibition curve (reference item).

Date : March 29, 2017



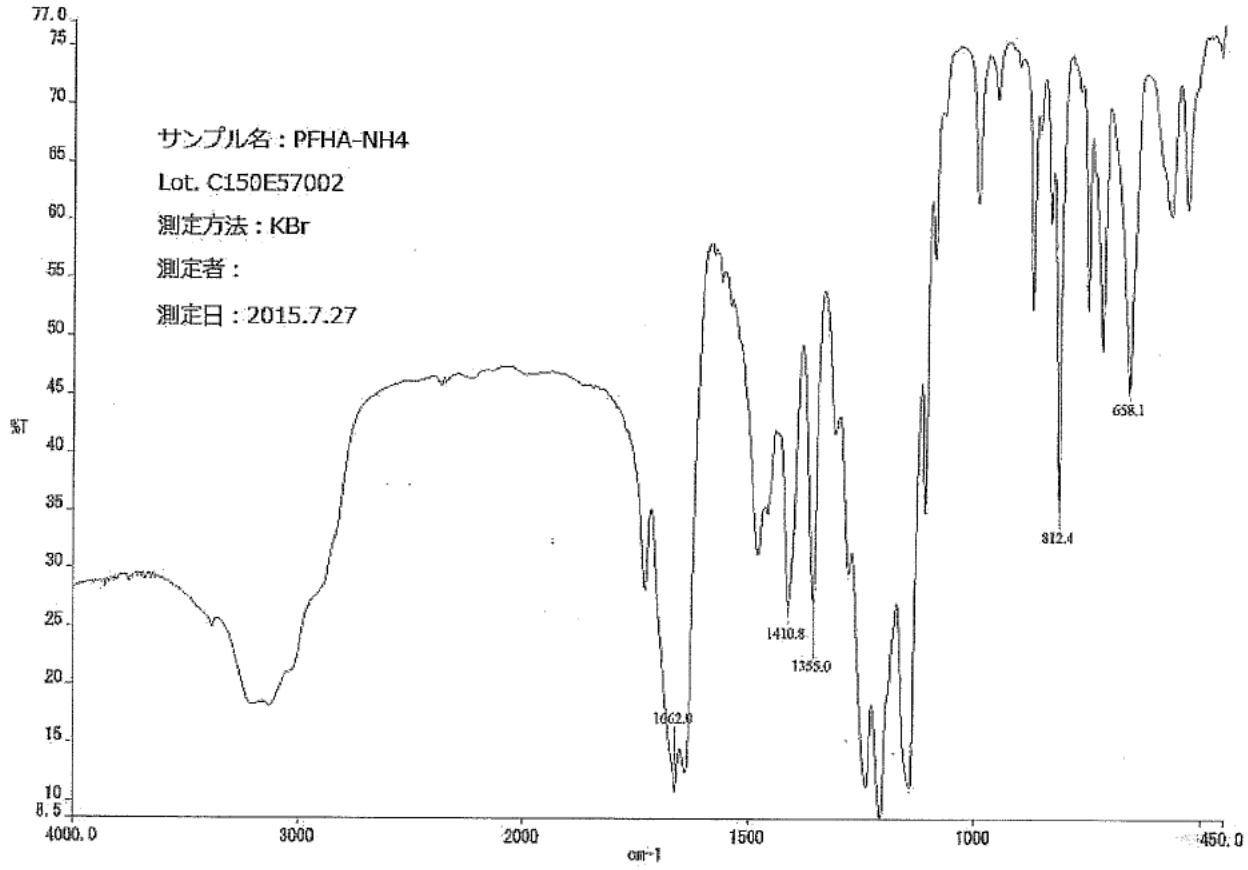
Instrument	: Shimadzu IRAffinity-1S
Study No.	: 97724
Sample	: Test item
Method	: KBr tablet
Date	: February 20, 2017
Name	:

Fig. 2 - 1 IR spectrum of test item measured before experimental start.



Instrument	: Shimadzu IRAffinity-1S
Study No.	: 97724
Sample	: Test item
Method	: KBr tablet
Date	: April 10, 2016
Name	:

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



IRスペクトル　PFHA-NH4

Reference 1　IR spectrum supplied by sponsor.