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TEST REPORT

Measuring concentration of APFHx in zebrafish embryos/larvae

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

Date March 18, 2019

Study Director

March, 2019

Chemicals Evaluation and Research Institute, Japan, Kurume

CONTENTS

1. Title

Measuring concentration of APFHx in zebrafish embryos/larvae

2. Sponsor

Name	DAIKIN INDUSTRIES, LTD.
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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

Measuring concentration of APFHx in zebrafish embryos/larvae to investigate characteristics of the uptake of APFHx to zebrafish embryos/larvae quantitatively.

5. Test method

This study was conducted in reference to a literature below.

Nawaji, T., Mizoguchi, N., Ono, M., Matuura, T., Seki, M. and Teraoka, H. (2018): Comparing time-series of chemical concentrations in zebrafish (*Danio rerio*) embryos/larvae exposed to teratogens with different hydrophobicity; caffeine, sodium valproate, and diethylstilbestrol, J. Toxicol. Sci., **43**, 267-273.

6. Date

Study initiation date	February 15, 2019
Experimental starting date	February 20, 2019
Experimental completion date	February 26, 2019
Study completion date	March 18, 2019

7. Personnel

Study Director

Study personnel (Biological study)

Study personnel (Analytical chemistry)

8. Approval of final report

Date March 18, 2019

Study Director

9. Summary

Test item

APFHx

Objective

Measuring concentration of APFHx in zebrafish embryos/larvae to investigate characteristics of the uptake of APFHx to zebrafish embryos/larvae quantitatively.

Test method

This study was conducted in reference to a literature below.

Nawaji, T., Mizoguchi, N., Ono, M., Matuura, T., Seki, M. and Teraoka, H. (2018): Comparing time-series of chemical concentrations in zebrafish (*Danio rerio*) embryos/larvae exposed to teratogens with different hydrophobicity; caffeine, sodium valproate, and diethylstilbestrol, J. Toxicol. Sci., **43**, 267-273.

Test condition

Test organism	Zebrafish (*Danio rerio*) embryo/larva
Dilution water	Reconstitution water (ISO6341-1982)
Test level	50.0, 25.0 and 10.0 mg/L as nominal concentration
Preparation of test solution	The test solution was prepared by using a stock solution prepared by mixing test item and dilution water and stirring.
Type of test	Static regime
Exposure duration	6 days
Replicate	
For measuring concentration in embryos/larvae	1 replicate/test level
For observation of symptoms	24 replicates/test level
Number of organism	
For measuring concentration in embryos/larvae	Exposure level: Total 180 embryos or larvae/test level Control: Total 90 embryos or larvae/test level (24 to 72 hpf*: 10 embryos or larvae /replicate/time point, 96 to 144 hpf : 5 embryos or larvae/replicate/time point) * hpf: hours post fertilization
For observation of symptoms	24 embryos or larvae/test level (1 embryo or larva/well)
Volume of test solution	
For measuring concentration in embryos/larvae	Approximately 500 mL/test level
For observation of symptoms	48 mL/test level (2 mL/well)
Temperature of test solution	28±1°C
Aeration	No aeration
pH adjustment	No adjustment
Lighting condition	Room light, 16-hour light/8-hour dark
Feeding	No feeding

Analysis of concentration of test item in test solution

For measuring the concentration in test solution

HPLC analysis (at the start and end of exposure)

For measuring the concentration in embryos/larvae

LC-MS analysis (every 24 hpf)

Result

Observation of test organism

144-hour NOEC (no observed effect concentration): 50 mg/L (nominal concentration)

Temporal behavior of test item concentrations in embryos/larvae

The concentrations of test item in all exposure levels reached maximum values at 96 hpf, i.e., 13.6, 31.1 and 48.7 mg/kg in the exposure level of 10.0, 25.0 and 50.0 mg/L, respectively.

After that, they gradually decreased to 40.4, 40.0 and 25.8% of maximum concentrations, i.e., 5.48, 12.5 and 12.6 mg/kg in the exposure level of 10.0, 25.0 and 50.0 mg/L at 144 hpf, respectively.

10. Test material

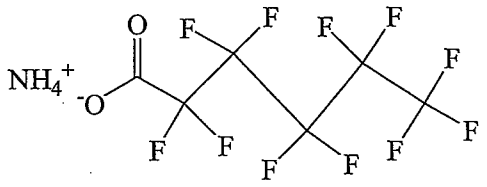
10.1 Test item

a) Chemical name etc.

Chemical name	2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt
Another name	APFHx
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	99.8%
Impurity	Water 0.2%
Supplier	DAIKIN INDUSTRIES, LTD.
Lot number	C150S1703

The test sample was treated as 100% in purity.

d) Physicochemical property

Water solubility	>500 g/L

e) Storage condition

The test sample was put into a bag containing silica gel, sealed and stored in a dark storage place at room temperature.

f) Safety and handling

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

10.2 Test organism

a) Parent fish used for egg collection

Species	Zebrafish (*Danio rerio*)
Reason for selection of species	Species determined by the sponsor
Supplier	A group bred and maintained in this test facility. The group's ancestor was supplied from National Institute for Environmental Studies, Japan.
Strain	NIES-R
Selection	Male and female fish whose histories until the definitive test were clear, and that matured enough to reproduce were selected.

Acclimation for spawning

Water	Dechlorinated tap water
Acclimation type	Flow-through regime
Dissolved oxygen concentration	At least 80% of air saturation value
Temperature	26±1°C
Lighting condition	Room light, 16-hour light/8-hour dark
Feed	Newly hatched *Artemia* nauplii (Salt Lake)
Feeding amount and frequency	Satiation amount, every day
Use of medicament for external disinfection	None
Aeration	Conducted

Pairing

Number of female and male	Two males and one female per container (Approximately 3 L)
Timing of pairing	The day of start of exposure
Water	Dechlorinated tap water
Temperature	26±1°C
Aeration	No aeration
Feed	No feeding during pairing

b) Fertilized eggs used for the study

Collection of fertilized eggs	Fertilized eggs were collected from three breeding groups, randomly selected and mixed.
Developmental stage	Fertilized eggs within 5 hours after fertilization (5 hpf)

11. Performance of definitive study

11.1 Dilution water

Reconstitution water (ISO6341-1982, OECD test guideline 203 Annex 2) was used.

11.2 Test apparatus and equipment

Test chamber

For measuring concentration in embryos/larvae

500 mL glass tank (IWAKI)

For observation of symptoms

Polystyrene 24-well plate (Sumitomo Bakelite)

Cover on test vessel

For measuring concentration in embryos/larvae

Transparent plastic lid

For observation of symptoms

Transparent polystyrene lid (sealed with paraffin film)

Microscope: Selection of embryos: Stereomicroscope SZ61TR (Olympus)
Observation of symptoms: Inverted microscope CKX53 (Olympus)

Incubator: Constant temperature incubator MIW-450V (AS ONE)

11.3 Preparation of test solution

The weighed test sample (0.10 g) and dilution water (1 L) were mixed, stirred and dissolved to prepare the stock solution of 100 mg/L (nominal). The required volume of the stock solution and dilution water were mixed and stirred to prepare the test solution.

11.4 Test condition

Type of test: Static regime (no renewal of test solution)

Exposure duration: 6 days

Test concentration: 50.0, 25.0 and 10.0 mg/L as nominal concentration
The test concentrations were decided from the results of the preliminary study and by consultation with the sponsor.
The results of the preliminary study are shown in Additional data.

Control: Dilution water without the test item

Replicate

For measuring concentration in embryos/larvae

1 replicate/test level

For observation of symptoms

24 replicates/test level

Number of test organism

For measuring concentration in embryos/larvae

Exposure level: Total 180 embryos or larvae/test level
Control: Total 90 embryos or larvae/test level
(24 to 72 hpf : 10 embryos or larvae /replicate/time point,
96 to 144 hpf : 5 embryos or larvae/replicate/time point)

For observation of symptoms

24 embryos or larvae/test level (1 embryo or larva/well)

Volume of test solution

For measuring concentration in embryos/larvae

Approximately 500 mL/test level

For observation of symptoms

48 mL/test level (2 mL/well)

Temperature of test solution

28±1°C

Aeration No aeration

pH adjustment No adjustment

Lighting condition Room light, 16-hour light/8-hour dark

Feeding No feeding

11.5 Observation and measurement of water quality

(Conducted in system for observation of symptoms)

a) Observation of test organism

The mortality, malformation and the other visible abnormality of the test organism were observed with inverted microscope every 24 hours from 24 hpf. The observation items are shown in a table below. Test organism showing coagulation of the embryo or no heartbeat were considered dead. Observations were performed on each test organism, and any positive outcome in one of the items in the table below (except for mortality) was considered abnormal.

Developmental stage	Observation item/organ	Example of developmental abnormality
Before hatching	-	Coagulation of the embryos (= mortality)
	Development	Development retardation
	Somite formation	Somite deformation, lack of somite
	Tail detachment	No detachment of tail
	Hatching	Delay of hatching
After hatching	-	Lack of heartbeat (= mortality)
	Eyes	Abnormality of eye formation
	Otic vesicle	Otic vesicle/otolith deformation
	Jaws	Lower jaw anomalies
	Body shape	Skeletal anomalies, kinked tail
	Fins	Fin (dorsal, caudal, pectoral, anal) deformation
	Yolk	Yolk shape anomalies
	Heart	Slow heartbeat, heart size anomalies
	Gut	Gut shape anomalies
	Blood circulation	Edema (e.g., eyes, ear, heart, yolk), blood accumulation, slowdown/lack of circulation (visible)
	Pigmentation	Deficiency/excess of pigmentation

b) Body weight of test organism

Wet body weights of five test organisms in the control were measured every 24 hpf. The dechorionated embryos or the larvae were weighed individually using an electronic balance (MSU6.6S-000-DM, Sartorius). The water on their body surfaces was removed with a filter paper prior to the measurement. The mean weights of the five organisms at each time point were used for converting the concentrations of test item in embryos/larvae (mg/kg).

c) Appearance of test solution

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

d) Condition of test solution

Item of measurement Dissolved oxygen concentration, pH and temperature

Frequency of measurement

At the start and end of exposure

Sample for measurement

The test solution for measurement was taken out from the test vessel.

Instrument Dissolved oxygen meter HQ30d (HACH)

pH meter HM-21P (DKK-TOA)

Thermometer of glass stick type

11.6 Measurement of test item concentration

(Conducted in system for measuring concentration in embryos/larvae)

a) Concentration of test item in test solution

Subject of measurement

All test levels

Frequency of measurement

At the start and end of exposure

Sample for measurement

The test solution was taken out from the middle layer of the test vessel.

Volume of sample Approximately 10 mL (all test levels)

Analytical condition Refer to Appendix 1

b) Concentration of test item in embryos/larvae

Subject of measurement

All test levels

Replicate Exposure level: 4 replicates/test level/time point

Control: 2 replicates/time point

Frequency of measurement

Every 24 hours from 24 hpf

Pretreatment and analytical method

Referred to Appendix 1

11.7 Calculation of mortality and developmental abnormality

(Conducted in system for observation of symptoms)

In each test level, mortality or developmental abnormality of all test organism were calculated. The percentage of mortality was calculated as the ratio of dead organisms to the number of embryos at the start of exposure (24 embryos). The percentage of developmental abnormalities was calculated as the ratio of abnormal organisms to the number of surviving embryos at 24 hpf.

11.8 Treatment of numerical value

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

12. Result and discussion

12.1 Mortality

Cumulative mortality of each observation time is shown in Table 1.

No dead embryos/larvae in the exposure level during exposure were confirmed. Number of dead embryo/larva in the control at the end of exposure was 0.

12.2 Observed morphological abnormalities

The morphological abnormalities observed during exposure are shown in Table 2.

No morphological abnormalities were observed in all test levels.

12.3 Body weight of test organism

Measured wet body weight of test organism in the control every 24 hpf are shown in Table 3.

The mean measured values at 24, 48, 72, 96, 120, 144 hpf were 0.239, 0.262, 0.294, 0.306, 0.311, 0.306 mg, respectively.

12.4 Observation and measurement of test solution

a) Appearance of test solution

The test solutions in all test levels were colorless and clear at the start and end of exposure.

b) Condition of test solution

Conditions of the test solutions are shown in Table 4.

The measured values of dissolved oxygen concentration, pH and temperature during exposure were 7.7-8.3 mg/L, 7.6-7.7 and 28.3-28.7°C, respectively.

c) Concentration of test item in test solution

The analytical method and results of measured concentrations of the test item are shown in Appendix 1. The calibration curve and chromatogram are shown in Appendix 2.

The measured concentrations of the test item in the test solutions at the start of exposure were 10.1 -48.8 mg/L (97.5-101% of the nominal concentrations), and those at the end of exposure were 10.4-50.7 mg/L (101-104% of the nominal concentrations). It was judged that the measured concentrations of test item maintained the nominal concentrations.

d) Concentration of test item in embryos/larvae

The analytical method and results of measured concentrations of the test item are shown in Appendix 1. The calibration curve and chromatogram are shown in Appendix 3.

The concentrations of test item in embryos/larvae as amount of test item per embryo/larva are shown in Table 5. The concentrations of test item in embryos/larvae as amount of test item per body weight are shown in Table6 and Figure 1. The values shown in Tables 5 and 6 were converted from values shown in Appendix table 1-2 with mean measured body weights and/or with the number of test organisms supplied to analysis, respectively.

The concentrations of test item in all exposure levels reached maximum values at 96 hpf, i.e., 13.6, 31.1 and 48.7 mg/kg in the exposure level of 10.0, 25.0 and 50.0 mg/L, respectively. After that, they gradually decreased to 40.4, 40.0 and 25.8% of maximum concentrations, i.e., 5.48, 12.5 and 12.6 mg/kg in the exposure level of 10.0, 25.0 and 50.0 mg/L at 144 hpf, respectively.

12.5 Discussion

This study measuring concentration of test item in zebrafish embryos/larvae was conducted to investigate characteristics of the uptake of the test item to the embryos/larvae quantitatively.

As a result, the concentration of test item in all exposure levels reached maximum values at 96 hpf and then gradually decreased to 25.8-40.4% of the maximum value at each test level.

The temporal behaviors were similar to data of diethylstilbestrol (DES) previously reported (Nawaji *et al.*, 2018). According to the report, DES is highly soluble in fat and it was considered that and the gradual decrease of concentrations of DES in embryos/larvae was due to a decline of total lipid concentration in whole embryos/larvae because of the energetic costs of development and growth. Although the test item is ammonium salt and has high water solubility, amphipathic property of test item may lead to the result of temporal behaviors similar to DES.

In construct, organogenesis of the zebrafish liver begins between 60-72 hpf and the liver becomes visible at 96 hpf (Chu and Sadler, 2009). Consequently, it was considered that much of metabolic function was supposed to be obtained at approximately 96 hpf and the activity in the liver may have been mainly responsible for the decline of concentration of test item in embryos/larvae. Additionally, activity of drug-metabolizing enzymes in the liver and/or in sites other than the liver might have partially contributed to the rapid decrease at 120 hpf and 144 hpf. No peaks other than test item were observed on the chromatogram obtained under the analytical conditions in this study. However, small amounts of metabolites might have been detected via additional detailed analyses.

No adverse effect on the test organisms were found in this study. The test item concentrations in the test solution maintained the nominal concentrations and the environmental conditions were within the suitable range. Therefore, this study was conducted under the appropriate condition to investigate characteristics of the uptake of the test item to the embryos/larvae quantitatively.

Reference:

Nawaji, T., Mizoguchi, N., Ono, M., Matuura, T., Seki, M. and Teraoka, H. (2018): Comparing time-series of chemical concentrations in zebrafish (*Danio rerio*) embryos/larvae exposed to teratogens with different hydrophobicity; caffeine, sodium valproate, and diethylstilbestrol, J. Toxicol. Sci., **43**, 267-273.

Chu, J. and Sadler, K.C. (2009): New school in liver development: lessons from zebrafish. Hepatology, **50**, 1656-1663.

13. Factor that affected the reliability of the test result

There were no factors which might have affected the reliability of the test.

Table 1 Cumulative mortality

Nominal concentration (mg/L)	Cumulative mortality (%)					
	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf
Control	0	0	0	0	0	0
10.0	0	0	0	0	0	0
25.0	0	0	0	0	0	0
50.0	0	0	0	0	0	0

Table 2 Observed morphological abnormalities

Nominal concentration (mg/L)	Result of observation (Left column: Number of affected zebrafish/Total survival number, Right column: Symptom detail)											
	24 hpf		48 hpf		72 hpf		96 hpf		120 hpf		144 hpf	
Control	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N
10.0	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N
25.0	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N
50.0	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N	0/24	N

N: Normal (No abnormal response)

Table 3 Wet body weight of test organism in the control

No.	Wet body weight (mg)					
	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf
1	0.247	0.235	0.262	0.307	0.310	0.302
2	0.230	0.269	0.295	0.305	0.308	0.300
3	0.238	0.270	0.308	0302	0.322	0.310
4	0.237	0.262	0.305	0.309	0.308	0.308
5	0.242	0.275	0.300	0.324	0.309	0.311
Mean	0.239	0.262	0.294	0.306	0.311	0.306
S.D.	0.006	0.016	0.019	0.003	0.006	0.005

Table 4 Condition of test solution

Nominal concentration (mg/L)	Dissolved oxygen concentration (mg/L)		pH		Temperature (°C)	
	At the start	At the end	At the start	At the end	At the start	At the end
Control	8.3	7.8	7.7	7.6	28.3	28.7
10.0	8.2	7.7	7.7	7.6	28.3	28.7
25.0	8.2	7.7	7.7	7.6	28.3	28.7
50.0	8.1	7.7	7.7	7.6	28.3	28.7

Table 5 Concentrations of test item in embryos/larvae (amount of test item per embryo/larva)

Nominal concentration (mg/L)	No.	24 hpf			48 hpf			72 hpf		
		Value	Mean	S.D.	Value	Mean	S.D.	Value	Mean	S.D.
Control	1	n.d.			n.d.			n.d.		
	2	n.d.			n.d.			n.d.		
10.0	1	0.366	0.359	0.026	1.10	1.23	0.12	2.75	2.64	0.14
	2	0.393			1.18			2.59		
	3	0.346			1.24			2.45		
	4	0.333			1.39			2.74		
25.0	1	0.639	0.722	0.068	1.64	2.27	0.43	5.44	5.72	0.22
	2	0.707			2.41			5.94		
	3	0.741			2.45			5.68		
	4	0.802			2.58			5.84		
50.0	1	1.09	1.25	0.14	4.23	4.33	0.15	10.2	10.4	0.3
	2	1.26			4.52			10.8		
	3	1.22			4.40			10.5		
	4	1.42			4.19			10.0		

Unit: ng/embryo or larva

n.d.: <0.200 ng/embryo or larva

Table 5 (continued) Concentrations of test item in embryos/larvae (amount of test item per embryo/larva)

Nominal concentration (mg/L)	No.	96 hpf			120 hpf			144 hpf		
		Value	Mean	S.D.	Value	Mean	S.D.	Value	Mean	S.D.
Control	1	n.d.			n.d.			n.d.		
	2	n.d.			n.d.			n.d.		
10.0	1	4.14	4.15	0.08	1.97	2.53	0.48	1.85	1.68	0.31
	2	4.21			3.09			1.75		
	3	4.03			2.70			1.88		
	4	4.20			2.34			1.22		
25.0	1	9.04	9.25	0.67	5.36	6.21	1.26	3.92	3.81	0.43
	2	10.0			7.93			4.22		
	3	9.52			5.18			3.92		
	4	8.44			6.37			3.20		
50.0	1	13.9	14.9	0.7	6.34	6.89	0.93	3.61	3.84	0.58
	2	15.5			5.87			3.32		
	3	15.4			7.58			3.79		
	4	14.7			7.76			4.66		

Unit: ng/embryo or larva

n.d.: <0.400 ng/embryo or larva

Table 6 Concentrations of test item in embryos/larvae (amount of test item per body weight)

Nominal concentration (mg/L)	No.	24 hpf			48 hpf			72 hpf		
		Value	Mean	S.D.	Value	Mean	S.D.	Value	Mean	S.D.
Control	1	n.d.[a]			n.d.[b]			n.d.[c]		
	2	n.d.[a]			n.d.[b]			n.d.[c]		
10.0	1	1.53	1.50	0.11	4.20	4.69	0.47	9.37	8.97	0.48
	2	1.65			4.50			8.82		
	3	1.45			4.75			8.35		
	4	1.39			5.32			9.33		
25.0	1	2.68	3.02	0.28	6.25	8.65	1.62	18.5	19.5	0.7
	2	2.96			9.17			20.2		
	3	3.10			9.33			19.3		
	4	3.36			9.83			19.9		
50.0	1	4.58	5.23	0.57	16.1	16.5	0.6	34.8	35.3	1.1
	2	5.28			17.2			36.6		
	3	5.13			16.8			35.8		
	4	5.95			16.0			34.1		

Unit: mg/kg

a: n.d.: <0.838 mg/kg

b: n.d.: <0.763 mg/kg

c: n.d.: <0.680 mg/kg

Table 6 (continued) Concentrations of test item in embryos/larvae (amount of test item per body weight)

Nominal concentration (mg/L)	No.	96 hpf			120 hpf			144 hpf		
		Value	Mean	S.D.	Value	Mean	S.D.	Value	Mean	S.D.
Control	1	n.d.[d]			n.d.[e]			n.d.[f]		
	2	n.d.[d]			n.d.[e]			n.d.[f]		
10.0	1	13.5	13.6	0.3	6.33	8.12	1.54	6.05	5.48	1.01
	2	13.8			9.92			5.72		
	3	13.2			8.68			6.15		
	4	13.7			7.53			3.99		
25.0	1	29.6	31.1	2.2	17.2	19.9	4.0	12.8	12.5	1.4
	2	32.7			25.5			13.8		
	3	31.1			16.6			12.8		
	4	27.6			20.5			10.4		
50.0	1	45.6	48.7	2.4	20.4	22.1	3.0	11.8	12.6	1.9
	2	50.6			18.8			10.8		
	3	50.5			24.4			12.4		
	4	48.0			24.9			15.2		

Unit: mg/kg

d: n.d.: <1.31 mg/kg

e: n.d.: <1.28 mg/kg

f: n.d.: <1.31 mg/kg

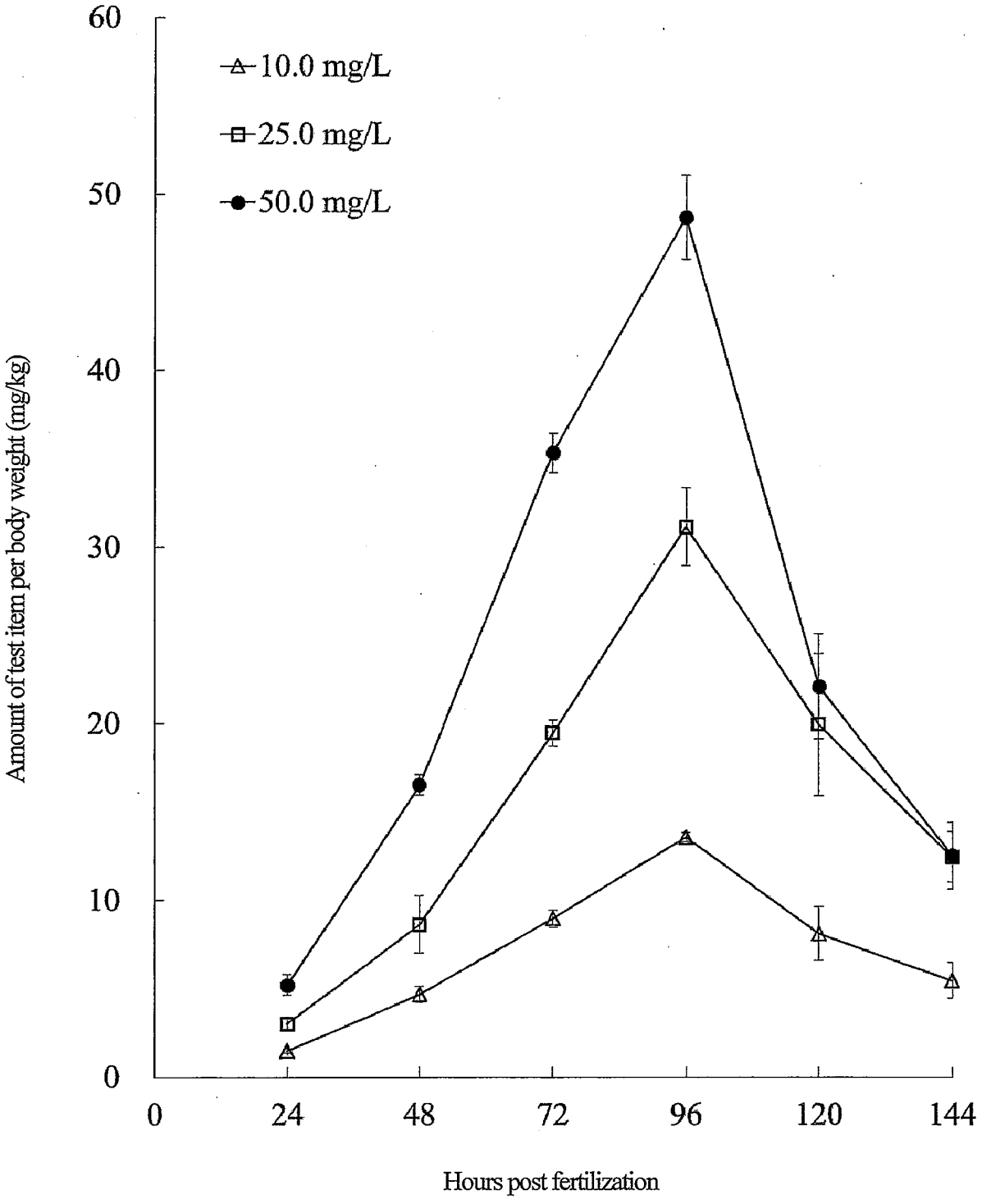


Figure 1 Concentrations of test item in embryos/larvae exposed to test solutions.

Appendix 1

Analytical method and measured concentration of test item

1. Analytical method of test item in test solution

1.1 Pretreatment of test solution

The collected test solutions were used as the high-performance liquid chromatography (HPLC) analytical samples after appropriate dilution with reconstituted water (ISO 6341-1982).

1.2 Determination of test item

a) Method of determination

Determination of the test item was conducted by an absolute calibration curve method using one concentration of standard solution.

In order to confirm the validity of this determination method, the calibration curve was made using four concentrations of standard solution 1.00, 5.00, 10.0 and 20.0 mg/L which were prepared in the same way described in c). As a result, the regression line of the calibration curve was a straight line from the origin. Therefore, the determination method was valid.

The drawn calibration curve and chromatograms which obtained by HPLC analysis of analytical sample are shown in Appendix 2.

The determination limit of the test item in the test solution was the lowest concentration of standard solution (1.00 mg/L) within the range of the calibration curve confirmed.

b) Analytical condition

Instrument	High-performance liquid chromatograph (instrument No. LC-166)
Pump	LC-20AD (Shimadzu)
UV-VIS detector	SPD-20AV (Shimadzu)
Column oven	CTO-20A (Shimadzu)
Auto injector	SIL-20AC (Shimadzu)
System controller	CBM-20A$_{VP}$ (Shimadzu)
Degasser	DGU-20A$_3$ (Shimadzu)
Column	L-column ODS (150 mm × 2.1 mm I.D., particle size 5 μm, Chemicals Evaluation and Research Institute, Japan)
Column temperature	40°C
Eluent	A (45%) : Acetonitrile B (55%) : Ultrapure water/0.5 mol/L tetra-*n*-butyl ammonium phosphate solution (100/1 v/v)
Flow rate	0.2 mL/min
Wave length	215 nm
Injection volume	20 μL

c) Preparation of standard solution and calculation of test item concentration

The test sample (50.0 mg) was precisely weighed by an electronic analytical balance. It was dissolved and filled up to 50 mL with ultrapure water to obtain 1000 mg/L solution of the test item. The solution was diluted with reconstituted water to prepare 10.0 mg/L standard solution.

The concentration of the test item in each HPLC analytical sample was determined on the basis of a comparison of the peak area on the chromatogram of the HPLC analytical sample solution with that of standard solution.

1.3 Results of measurement

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

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

Nominal concentration (mg/L)	Measured concentration (mg/L) (Percentage of measured concentration versus nominal concentration %)		
	At the start	At the end	Geometric mean
Control	n.d.	n.d.	
10.0	10.1 (101)	10.4 (104)	10.3 (103)
25.0	24.7 (99.0)	25.6 (103)	25.2 (101)
50.0	48.8 (97.5)	50.7 (101)	49.7 (99.5)

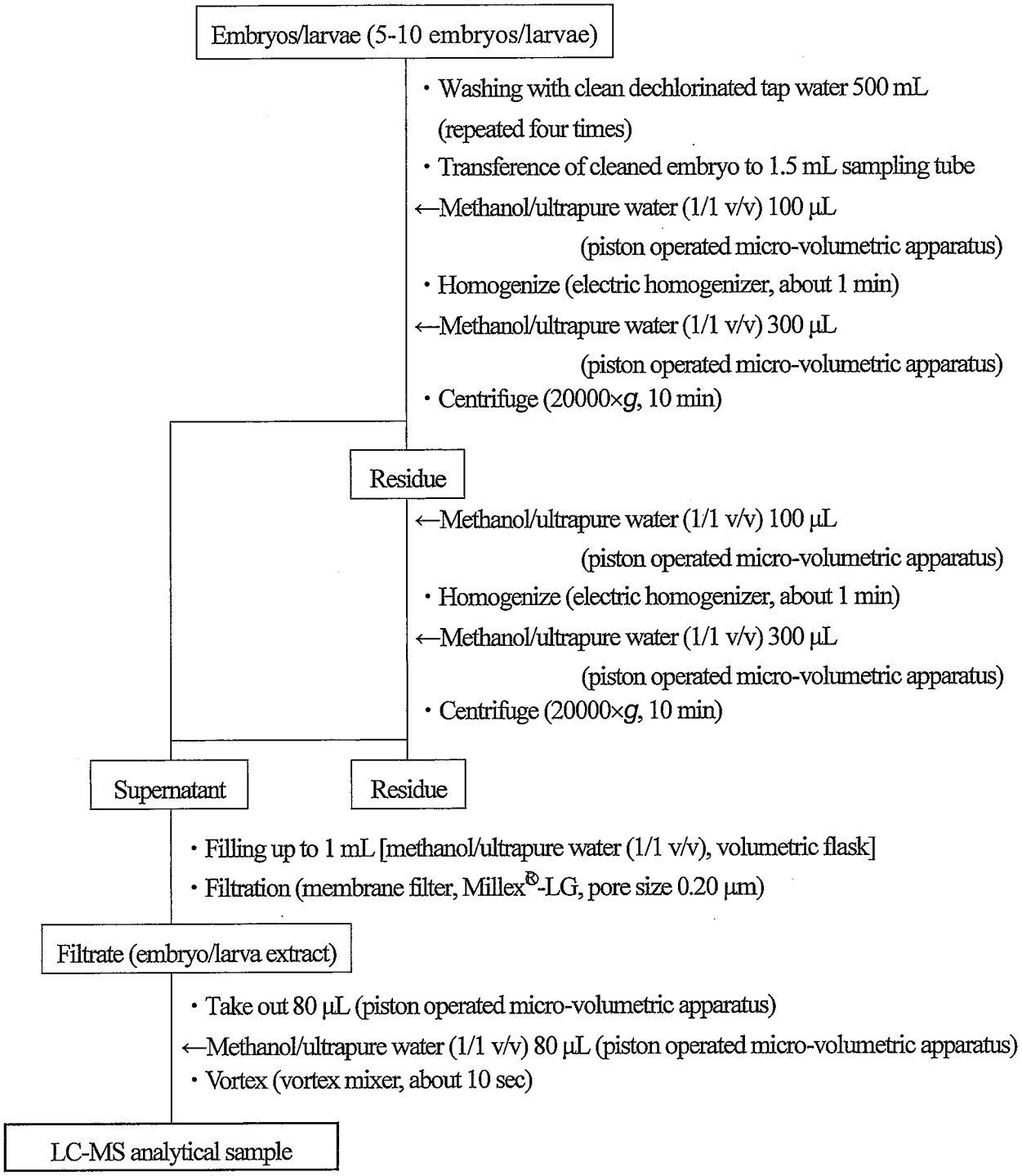
n.d. : <1.00 mg/L

2. Analytical method of test item in embryos/larvae

2.1 Pretreatment of embryos/larvae

The collected embryos/larvae in test vessel were pretreated according to the following flow scheme to prepare the liquid chromatography-mass spectrometry (LC-MS) analytical samples.

Flow scheme



2.2 Determination of test item

a) Method of determination

Determination of the test item was conducted by an absolute calibration curve method using one concentration of standard solution.

In order to confirm the validity of this determination method, the calibration curve was made using five concentrations of standard solution 0.00200, 0.00400, 0.0100, 0.0500 and 0.100 mg/L which were prepared in the same way described in c). As a result, the regression line of the calibration curve was a straight line from the origin. Therefore, the determination method was valid (operated in study No. 98280).

The drawn calibration curve and chromatograms which obtained by LC-MS analysis of some analytical samples are shown in Appendix 3.

The determination limit of the test item in the test solution was the lowest concentration of standard solution (0.00200 mg/L) within the range of the calibration curve confirmed.

b) Analytical condition

Instrument	Liquid chromatograph-mass spectrometer (instrument No. LCMS-014)
Liquid chromatograph	Nexera X2 (Shimadzu)
Mass spectrometer	LCMS-8060 (Shimadzu)

Liquid chromatograph condition

Column	L-column2 ODS (150 mm × 2.1 mm I.D., particle size 5 μm, Chemicals Evaluation and Research Institute, Japan)
Column temperature	40°C
Eluent	A (40%): 5 mmol/L ammonium acetate solution B (60%): 5 mmol/L ammonium acetate solution in methanol
Flow rate	0.2 mL/min
Injection volume	5 μL

Mass condition

Ionization mode	Electrospray ionization (ESI)
Detection ion	Negative
Detection mode	Selected ion monitoring (SIM)
Measurement ion (*m/z*)	313.1
Interface temperature	300°C
Desolvation temperature	240°C
Nebulizer gas	1.50 L/min
Drying gas	10.00 L/min

c) Preparation of standard solution and calculation of test item concentration

1000 mg/L solution of the test item preparing in section 1.2 c) was diluted with methanol/ultrapure water (1/1 v/v) to prepare a stock standard solution of 0.0200 mg/L. Additionally, the solution was diluted with control embryos/larvae extract to prepare a standard solution of 0.0100 mg/L after dilution to produce the solutions whose ratio were methanol/ultrapure water/ control embryos/larvae extract (1/1/2 v/v/v).

The concentration of the test item in each LC-MS analytical sample was determined on the basis of a comparison of the peak area on the chromatogram of the LC-MS analytical sample solution with that of standard solution.

2.3 Results of measurement

The results of the measured concentrations of the test item in the embryos/larvae are shown below.

Appendix table 1-2 Measured concentrations of test item in embryos/larvae

Nominal concentration (mg/L)	Measured concentration (mg/L)					
	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf	144 hpf
Control	0.0000590*	0.000166*	0.0000482*	0.0000390*	0.0000251*	0.0000815*
10.0	0.00359	0.0123	0.0264	0.0207	0.0126	0.00839
25.0	0.00722	0.0227	0.0572	0.0463	0.0311	0.0191
50.0	0.0125	0.0433	0.104	0.0744	0.0344	0.0192

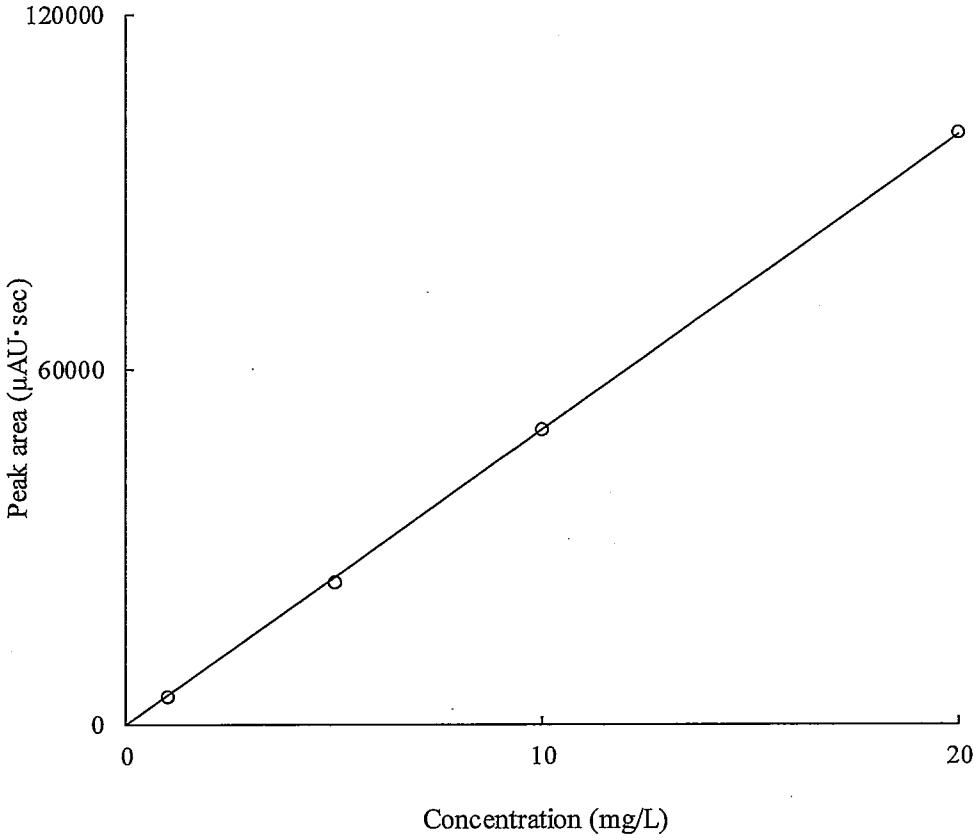
* n.d. : <0.00200 mg/L

24 to 72 hpf: 10 embryos/larvae were supplied.

96 to 144 hpf: 5 embryos/larvae were supplied.

Appendix 2

Calibration curve and chromatogram
on measuring concentration of test item in test solution



y = 4976x

r = 1.00

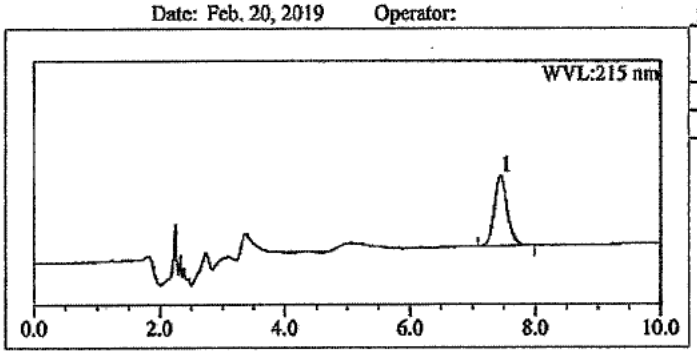
Concentration (mg/L)	Peak area (μAU·sec)
1.00	4587
5.00	24036
10.0	49535
20.0	99856

Appendix figure 2-1 Calibration curve of test item for analysis by HPLC.

98608

Standard solution 10.0 mg/L

Date: Feb. 20, 2019 Operator:

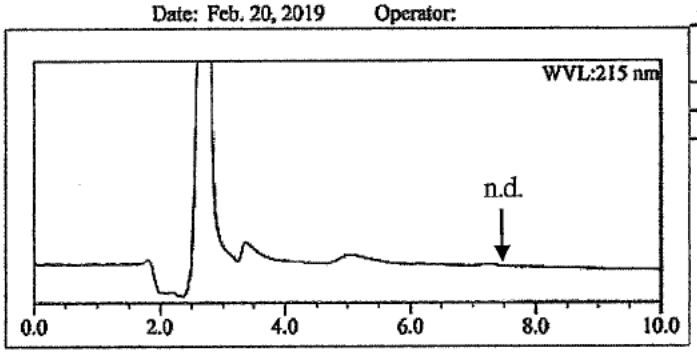


98608 190220 S2

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.45	3458	49107	100.00
Total	-	-	49107	100.00

Control

Date: Feb. 20, 2019 Operator:

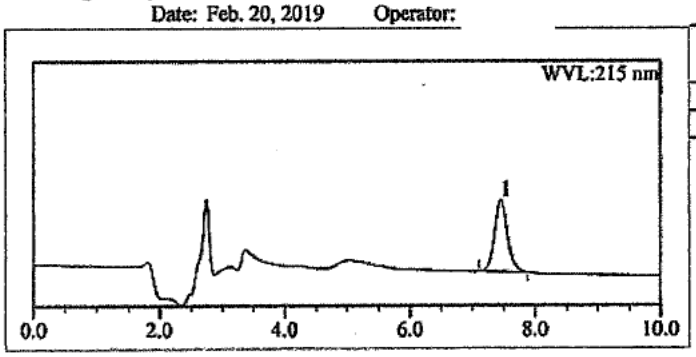


98608 190220 H0hZ

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	-	-	-	-
Total	-	-	0	0.00

10.0 mg/L exposure level

Date: Feb. 20, 2019 Operator:

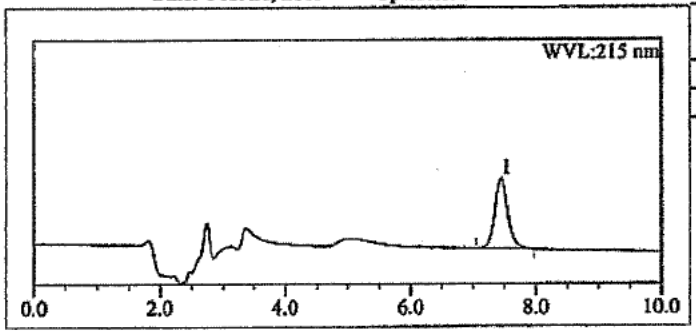


98608 190220 H0hC

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.45	3506	49781	100.00
Total	-	-	49781	100.00

25.0 mg/L exposure level

Date: Feb. 20, 2019 Operator:



98608 190220 H0hB

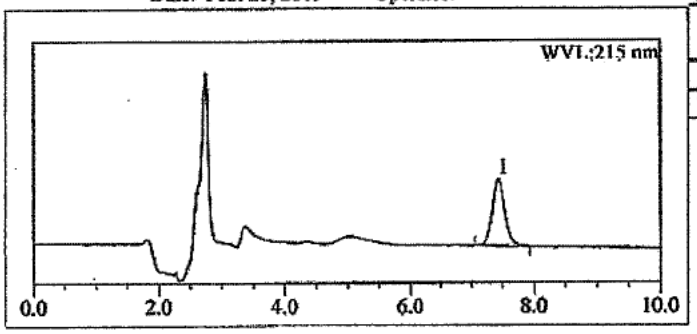
No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.45	3424	48600	100.00
Total	-	-	48600	100.00

Appendix figure 2-2 HPLC chromatograms at start of exposure.

98608

50.0 mg/L exposure level

Date: Feb. 20, 2019 Operator:



98608 190220 H0hA

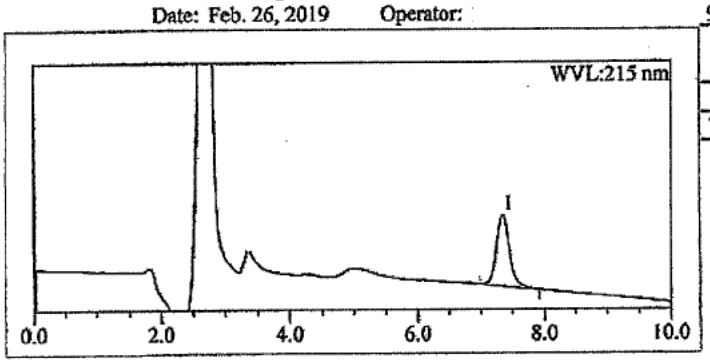
No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.43	3415	47898	100.00
Total	-	-	47898	100.00

Appendix figure 2-2 (Continued) HPLC chromatograms at start of exposure.

98608

Standard solution 10.0 mg/L

Date: Feb. 26, 2019 Operator:

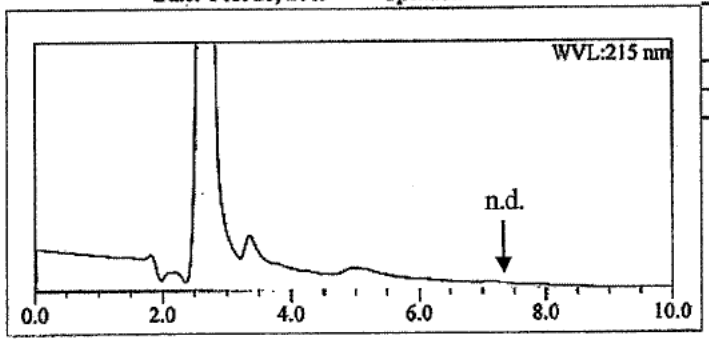


98608 190226 S2

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.34	3505	49484	100.00
Total	-	-	49484	100.00

Control

Date: Feb. 26, 2019 Operator:

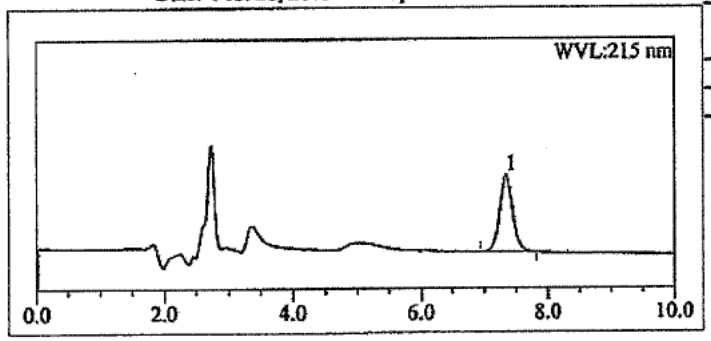


98608 190226 H144hZ

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	-	-	-	-
Total	-	-	0	0.00

10.0 mg/L exposure level

Date: Feb. 26, 2019 Operator:

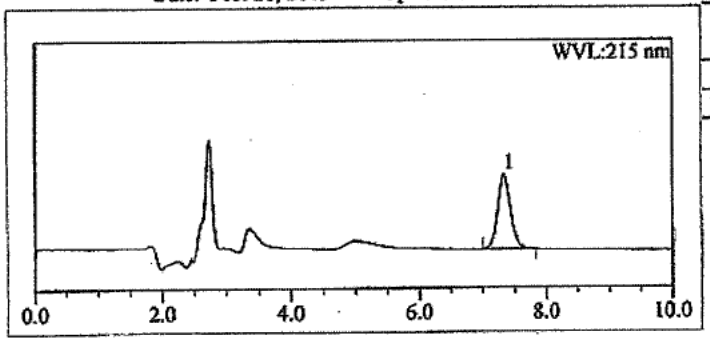


98608 190226 H144hC

No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.34	3681	51559	100.00
Total	-	-	51559	100.00

25.0 mg/L exposure level

Date: Feb. 26, 2019 Operator:



98608 190226 H144hB

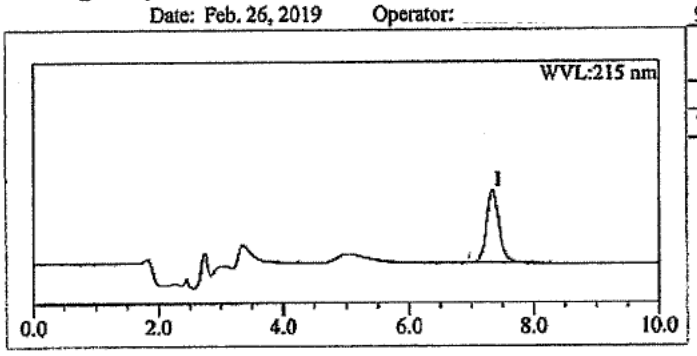
No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.33	3657	50756	100.00
Total	-	-	50756	100.00

Appendix figure 2-3 HPLC chromatograms at end of exposure.

98608

50.0 mg/L exposure level

Date: Feb. 26, 2019 Operator:



98608 190226 H144hA

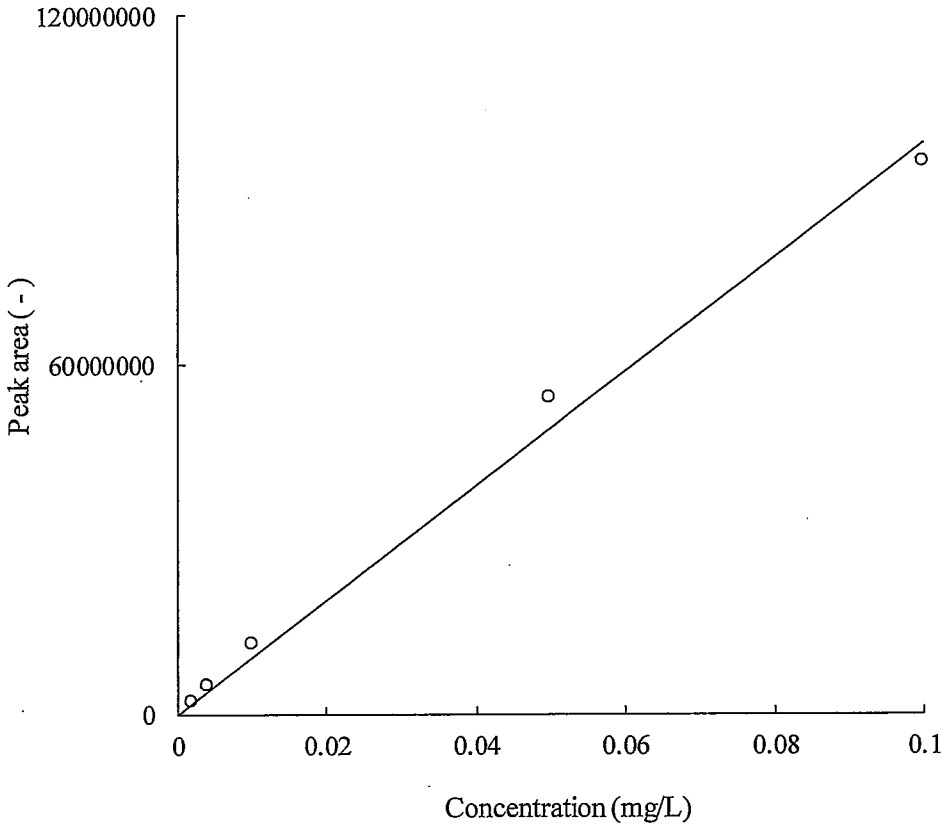
No.	Time (min)	Height (μAU)	Area (μAU·sec)	Area (%)
1	7.34	3605	50186	100.00
Total	-	-	50186	100.00

Appendix figure 2-3 (Continued) HPLC chromatograms at end of exposure.

Appendix 3

Calibration curve and chromatogram
on measuring concentration of test item in embryos/larvae

Study No. 98280[B+S]



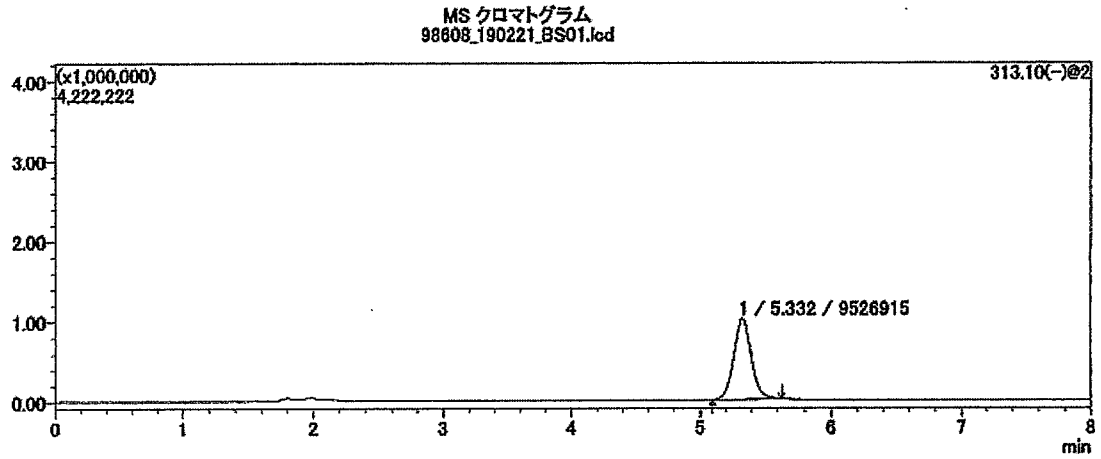
y = 980204528x

r = 0.996

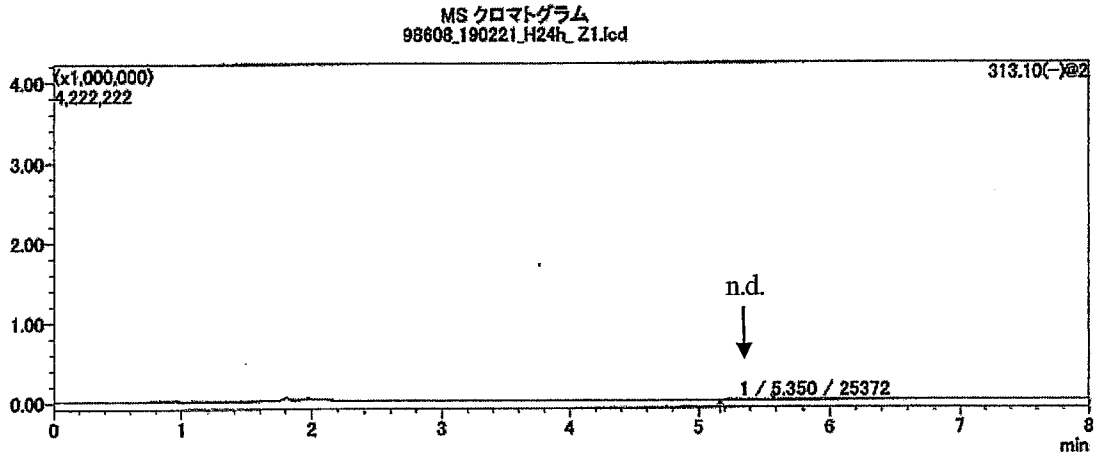
Concentration (mg/L)	Peak area (-)
0.00200	2399414
0.00400	5020360
0.0100	12224342
0.0500	54616523
0.100	94922313

Appendix figure 3-1 Calibration curve of test item for analysis by LC-MS (operated in study No. 98280).

サンプルID	: 98608
サンプル名	: BS_Standard solution 0.0100 mg/L
バイアル番号	: 3
分析日	: 2019/02/21
注入量	: 5
データファイル	: 98608_190221_BS01.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

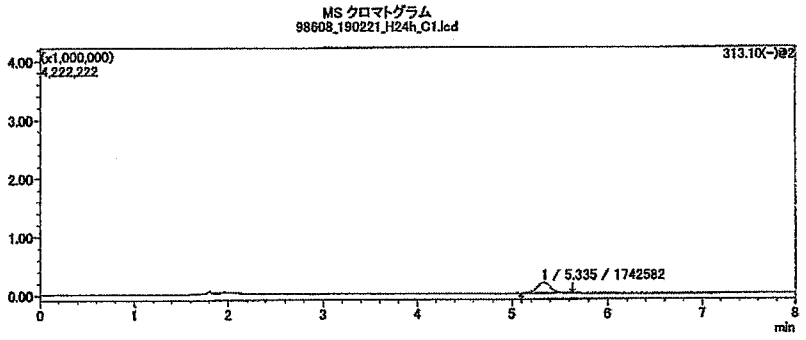


サンプルID	: 98608
サンプル名	: Control-1
バイアル番号	: 4
分析日	: 2019/02/21
注入量	: 5
データファイル	: 98608_190221_H24h_ Z1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

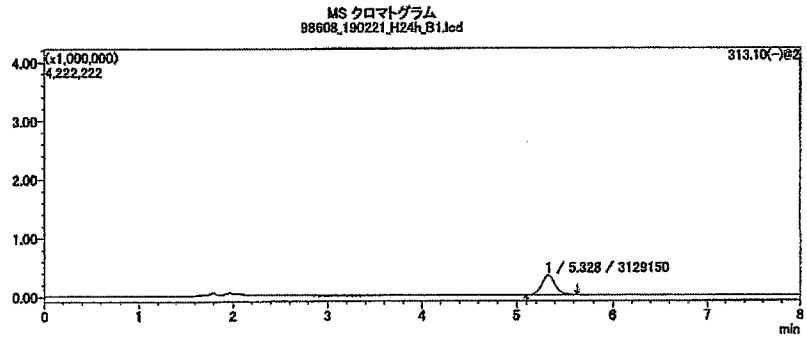


Appendix figure 3-2　LCMS chromatograms at 24 hours after exposure.

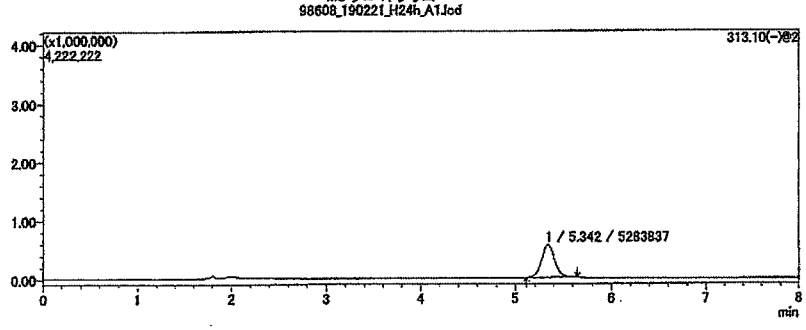
サンプルID	: 98608
サンプル名	: 10.0 mg/L exposure level-1
バイアル番号	: 6
分析日	: 2019/02/21
注入量	: 5
データファイル	: 98608_190221_H24h_C1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



サンプルID	: 98608
サンプル名	: 25.0 mg/L exposure level-1
バイアル番号	: 10
分析日	: 2019/02/21
注入量	: 5
データファイル	: 98608_190221_H24h_B1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

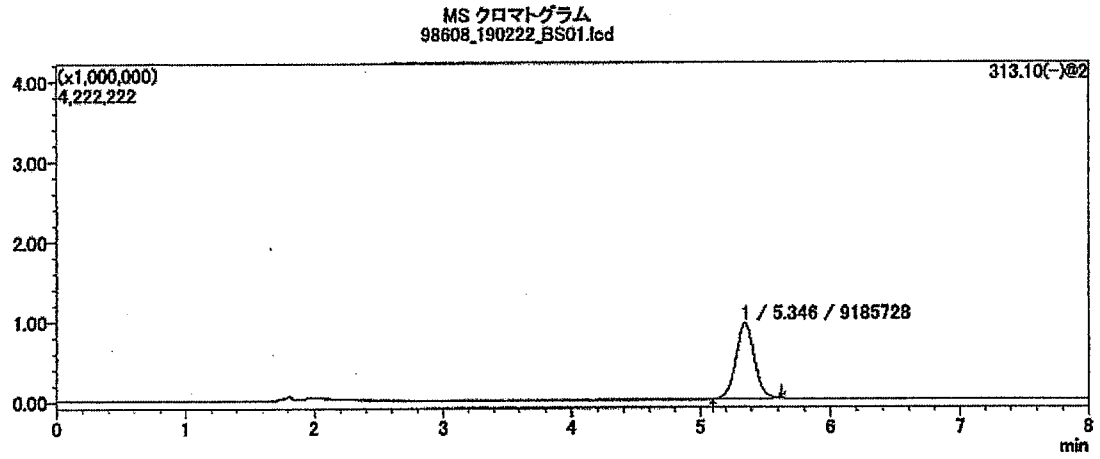


サンプルID	: 98608
サンプル名	: 50.0 mg/L exposure level-1
バイアル番号	: 14
分析日	: 2019/02/21
注入量	: 5
データファイル	: 98608_190221_H24h_A1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

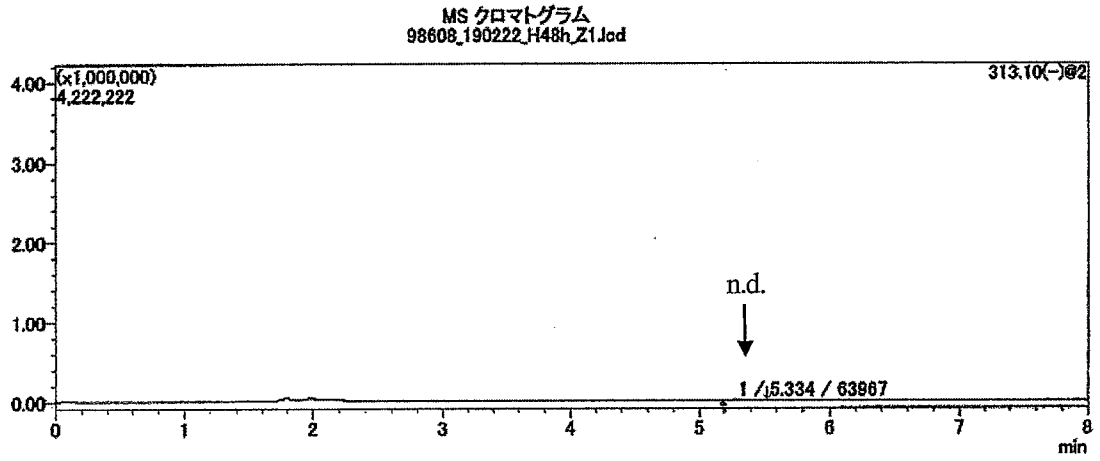


Appendix figure 3-2 (Continued)　LCMS chromatograms at 24 hours after exposure.

サンプルID	: 98608
サンプル名	: BS_Standard solution 0.0100 mg/L
バイアル番号	: 3
分析日	: 2019/02/22
注入量	: 5
データファイル	: 98608_190222_BS01.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



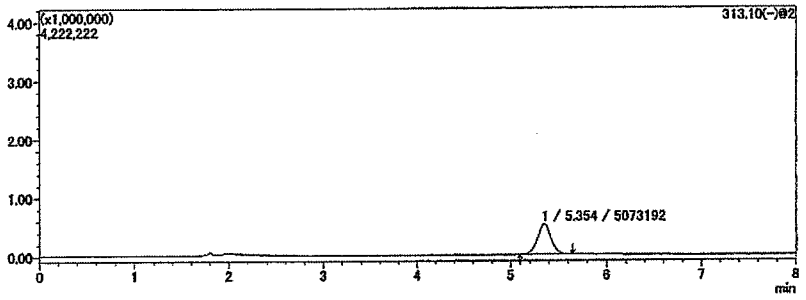
サンプルID	: 98608
サンプル名	: Control-1
バイアル番号	: 4
分析日	: 2019/02/22
注入量	: 5
データファイル	: 98608_190222_H48h_Z1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



Appendix figure 3-3 LCMS chromatograms at 48 hours after exposure.

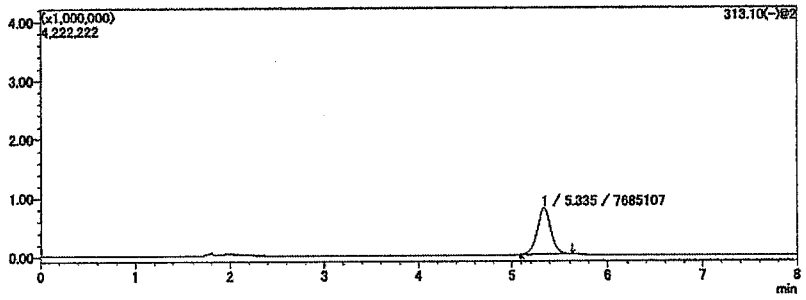
サンプルID	: 98608
サンプル名	: 10.0 mg/L exposure level-1
バイアル番号	: 6
分析日	: 2019/02/22
注入量	: 5
データファイル	: 98608_190222_H48h_C1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190222_H48h_C1.lcd



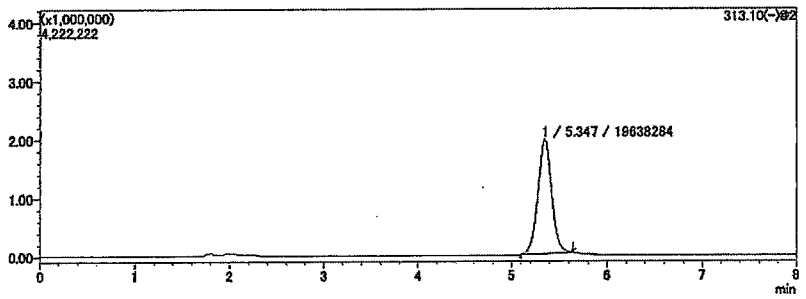
サンプルID	: 98608
サンプル名	: 25.0 mg/L exposure level-1
バイアル番号	: 10
分析日	: 2019/02/22
注入量	: 5
データファイル	: 98608_190222_H48h_B1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190222_H48h_B1.lcd



サンプルID	: 98608
サンプル名	: 50.0 mg/L exposure level-1
バイアル番号	: 14
分析日	: 2019/02/22
注入量	: 5
データファイル	: 98608_190222_H48h_A1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

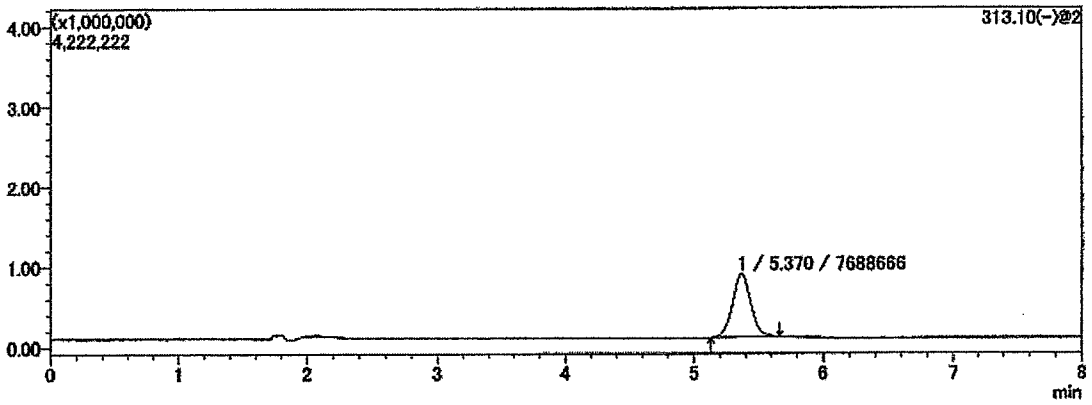
MS クロマトグラム
98608_190222_H48h_A1.lcd



Appendix figure 3-3 (Continued)　LCMS chromatograms at 48 hours after exposure.

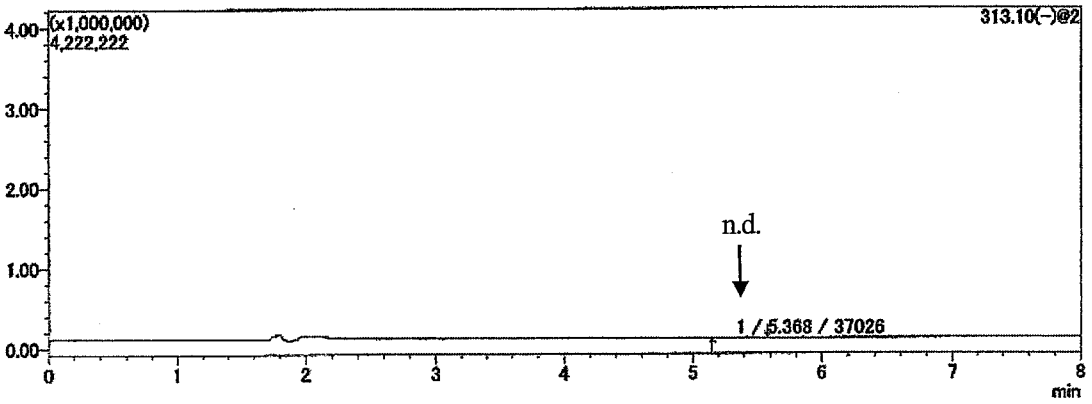
サンプルID	: 98608
サンプル名	: BS_Standard solution 0.0100 mg/L
バイアル番号	: 3
分析日	: 2019/02/23
注入量	: 5
データファイル	: 98608_190223_BS01.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190223_BS01.lcd



サンプルID	: 98608
サンプル名	: Control-1
バイアル番号	: 4
分析日	: 2019/02/23
注入量	: 5
データファイル	: 98608_190223_H72h_Z1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

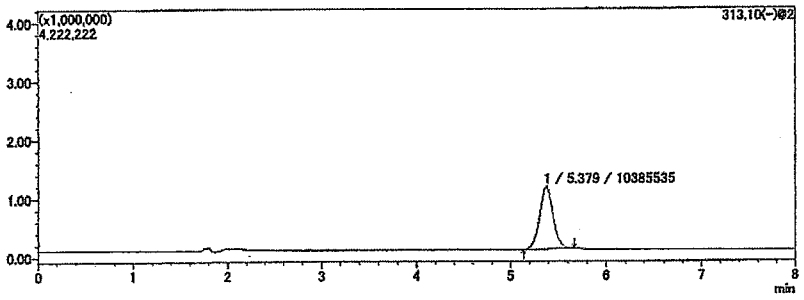
MS クロマトグラム
98608_190223_H72h_Z1.lcd



Appendix figure 3-4 LCMS chromatograms at 72 hours after exposure.

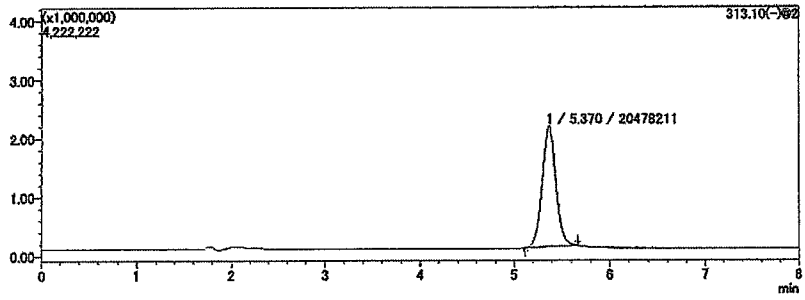
サンプルID	: 98608
サンプル名	: 10.0 mg/L exposure level-1
バイアル番号	: 6
分析日	: 2019/02/23
注入量	: 5
データファイル	: 98608_190223_H72h_C1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190223_H72h_C1.lcd



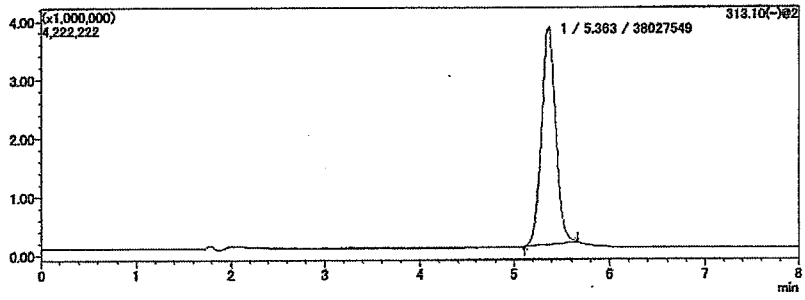
サンプルID	: 98608
サンプル名	: 25.0 mg/L exposure level-1
バイアル番号	: 10
分析日	: 2019/02/23
注入量	: 5
データファイル	: 98608_190223_H72h_B1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190223_H72h_B1.lcd



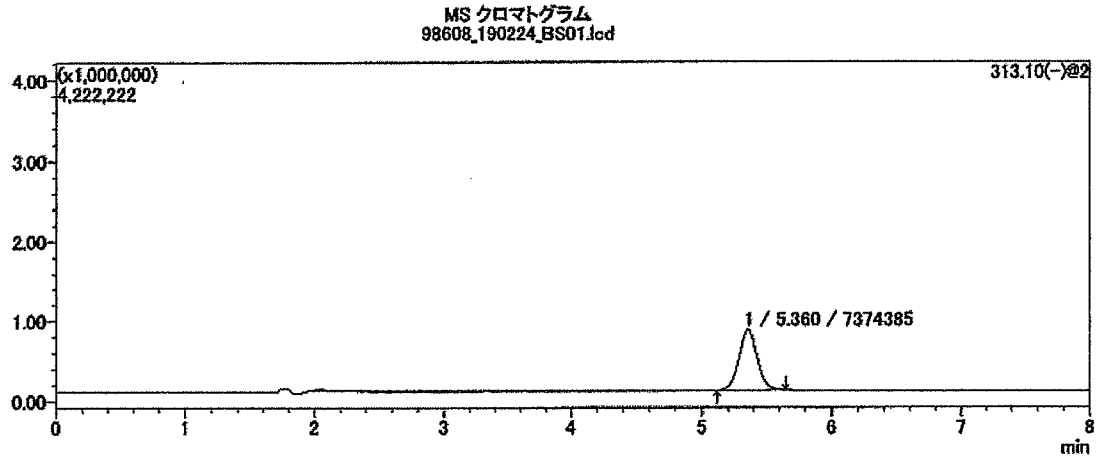
サンプルID	: 98608
サンプル名	: 50.0 mg/L exposure level-1
バイアル番号	: 14
分析日	: 2019/02/23
注入量	: 5
データファイル	: 98608_190223_H72h_A1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

MS クロマトグラム
98608_190223_H72h_A1.lcd

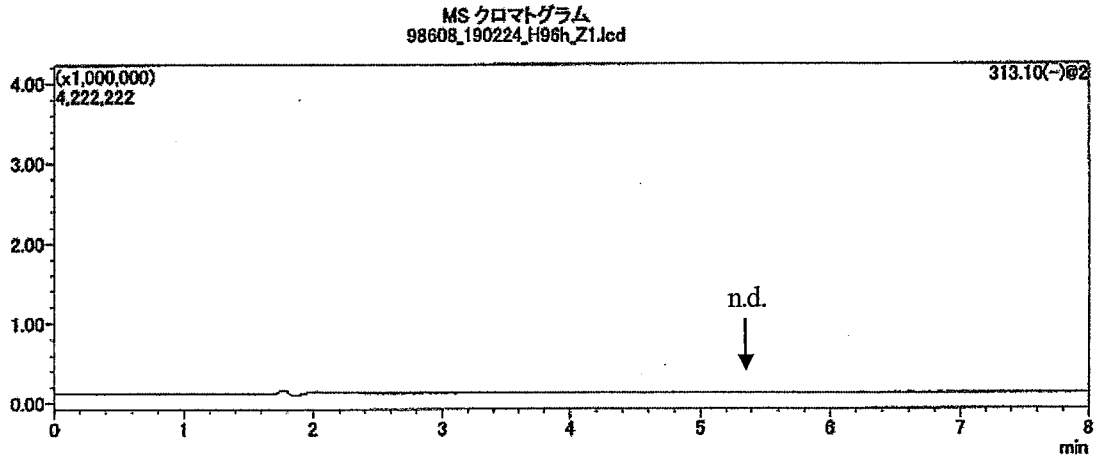


Appendix figure 3-4 (Continued)　LCMS chromatograms at 72 hours after exposure.

サンプルID	: 98608
サンプル名	: BS_Standard solution 0.0100 mg/L
バイアル番号	: 3
分析日	: 2019/02/24
注入量	: 5
データファイル	: 98608_190224_BS01.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

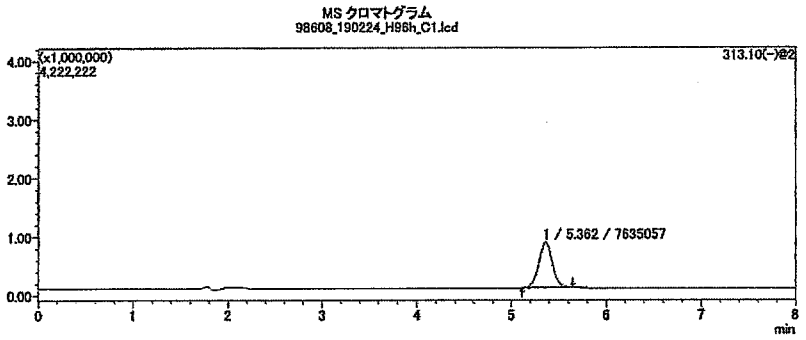


サンプルID	: 98608
サンプル名	: Control-1
バイアル番号	: 4
分析日	: 2019/02/24
注入量	: 5
データファイル	: 98608_190224_H96h_Z1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm

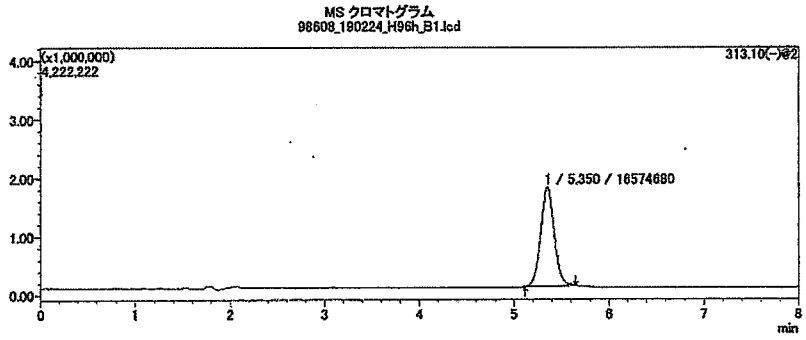


Appendix figure 3-5 LCMS chromatograms at 96 hours after exposure.

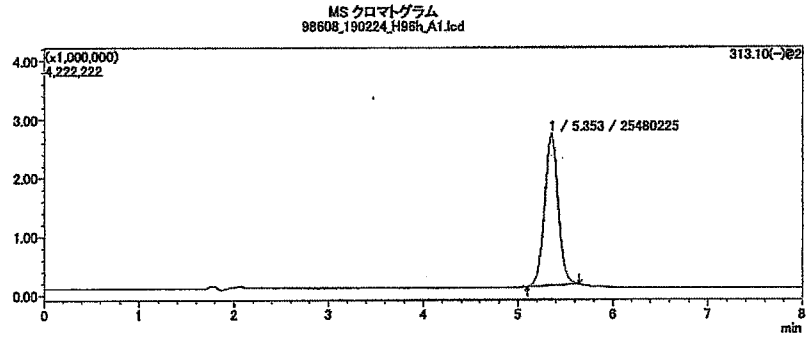
サンプルID	: 98608
サンプル名	: 10.0 mg/L exposure level-1
バイアル番号	: 6
分析日	: 2019/02/24
注入量	: 5
データファイル	: 98608_190224_H96h_C1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



サンプルID	: 98608
サンプル名	: 25.0 mg/L exposure level-1
バイアル番号	: 10
分析日	: 2019/02/24
注入量	: 5
データファイル	: 98608_190224_H96h_B1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



サンプルID	: 98608
サンプル名	: 50.0 mg/L exposure level-1
バイアル番号	: 14
分析日	: 2019/02/24
注入量	: 5
データファイル	: 98608_190224_H96h_A1.lcd
分析時のメソッドファイル	: 98608_98280_sim180627.lcm



Appendix figure 3-5 (Continued)　LCMS chromatograms at 96 hours after exposure.

Additional data

Result of preliminary study

1. Measuring concentration of test item in embryos/larvae

Type of test	Static regime
Stage of the test organism	≤5 hpf
Test level	50.0, 10.0 mg/L and a control
Replicate	Exposure level: 2 replicates/test level/time point Control: 1 replicate/time point
Number of test organism	24 and 72 hpf: 10 embryos or larvae/replicate 144 hpf: 5 larvae/replicate
Preparation of test solution	The test solution was prepared by using a stock solution prepared by mixing test item and dilution water [reconstituted water (ISO 6341-1982: described in OECD TG 203 Annex 2)] and stirring.
Analysis	The concentration of the test item in test solution (only at the start and end of exposure) and the concentration of test item in embryos/larvae (24, 72 and 144 hpf) were measured.

<The results of measuring test item concentration in test solution>

Test level (mg/L)	Measured concentration (mg/L) [% to nominal concentration]	
	At the start of exposure	At the end of exposure
Control	n.d.	n.d.
10.0	10.2 [102]	10.1 [101]
50.0	50.5 [101]	49.9 [99.9]

n.d.: <1.00 mg/L

<The results of measuring test item concentration in embryos/larvae>

Test level (mg/L)	Concentration in embryos/larvae (ng/embryo or larva)			Concentration in embryos/larvae (mg/kg)*1		
	24 hpf	72 hpf	144 hpf	24 hpf	72 hpf	144 hpf
Control	n.d.*2	n.d.*2	n.d.*3	n.d.*4	n.d.*5	n.d.*6
10.0	0.359	2.31	3.74	1.39	7.55	11.5
50.0	1.37	7.71	11.7	5.31	25.2	36.2

*1 The values were calculated using data of each body weight at 24, 72 and 144 hpf measured in the definitive study of study number 98280.

*2 n.d.: <0.200 ng/embryo or larva

*3 n.d.: <0.400 ng/larva

*4 n.d.: <0.773 mg/kg

*5 n.d.: <0.654 mg/kg

*6 n.d.: <1.24 mg/kg

2. Condition of definitive study

Nominal concentration of test solution

50.0, 25.0, 10.0 mg/L and a control

Type of test: Static regime

Replicate: Exposure level: 4 replicates/test level/time point

Control: 2 replicates/test level/time point

Number of test organism for measurement

0 to 72 hpf: 10 embryos or larvae/replicate

96 to 144 hpf: 5 embryos or larvae/replicate

Washing of body surface

The body surface was washed by repeating the operation that the test organism was transferred to different container containing approximately 500 mL of fresh dechlorinated tap water for four times.

Frequency of concentration measurement

Measurement of concentration in embryos/larvae: every 24 hours from 24 hpf

Measurement of concentration in test solution: at the start and end of exposure