STUDY CODE	937-17-V-0550

FINAL REPORT

EVALUATION OF SKIN SENSITIZATION POTENCY OF PFHxA-NH4 BASED ON EC3 VALUE DERIVED FROM LOCAL LYMPH NODE ASSAY (LLNA)

June, 2017

Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute, Japan

Study Code: 937-17-V-0550

TITLE

Evaluation of skin sensitization potency of PFHxA-NH4 based on EC3 value derived from local lymph node assay (LLNA)

SPONSOR

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

TESTING FACILITY

Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute (CERI), Japan
1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama 345-0043, Japan

PURPOSE OF STUDY

The purpose of this study was to evaluate the skin sensitization potential of the test chemicals.

METHOD OF STUDY

This study was conducted in accordance with the OECD TG429, then EC3 value was calculated and skin sensitization potency of test chemical was estimated by the criterion of Kimber, I.et al (2003).

PERIOD OF STUDY

Commencement of Study:	April 26, 2017
Animal Receipt:	April 20, 2017
Commencement of Pre-screen test:	April 26, 2017
Sensitization period:	May 4 – 6, 2017
Administration of ^{3}H-methyl thymidine:	May 9, 2017
Collection of lymph nodes:	May 9, 2017
Measurement of ^{3}H-methyl thymidine incorporation:	May 10, 2017
Completion of Study:	June 1, 2017

NAMES, ASSIGNED SECTIONS AND JOB ASSIGNMENT OF STUDY DIRECTOR AND PERSONNEL

Study Director:

Chemicals Assessment and Research Center

Study Staff

Chemicals Assessment and Research Center

Certified radiation protection supervisor

Chemicals Assessment and Research Center

Experimental Animal Manager

Chemicals Assessment and Research Center

SUMMARY

Skin sensitization potential of PFHxA-NH4 was evaluated by Local Lymph Node Assay (LLNA). Study was conducted in accordance with the OECD TG429. Test solutions, 25%, 50%, and 90 % of PFHxA-NH4 was applied to the dorsum of both ears of female CBA/J mice in 9 weeks of age daily for three consecutive days, then ^{3}H-methyl thymidine was injected into all mice via the tail vein and incorporation of ^{3}H-methyl thymidine into auricular lymph node cells was measured. Stimulation Indices (SI) of 50 % of PFHxA-NH4 exceeded 3, this chemical was evaluated as positive in LLNA. Since EC3 of PFHxA-NH4 was 43.3, this chemical was classified as weak sensitizer. GHS sub-category of PFHxA-NH4 was classified 1B.

While, the known human contact allergens, α-Hexylcinnamaldehyde (HCA) used as positive control showed clear positive response with SI value of 13.9 (SI >3). Therefore it confirms the validity of this study.

MATERIALS AND METHODS

1 TEST SUBSTANCES AND VEHICLE

1.1 Test substance

1) Test substance name

2,2,3,3,4,4,5,5,6,6,6-undecafluorohexanoic acid, ammonium salt

Synonym

PFHxA-NH4

2) CAS No.

21615-47-4

3) Supplier

DAIKIN INDUSTRIES, LTD.

4) Lot No.

C15FD57002

5) Purity

99.8 %

6) Appearance in normal temperature

White powder

7) Storage Condition

Test substance was stored with protection from light at room temperature.

8) Handling precautions

Gloves, a mask, a head cap and a lab coat were worn when handling.

1.2 Positive control substance

1) Chemical name

α-Hexylcinnamaldehyde (HCA)

CAS No. 101-86-0

2) Lot No.

SAF6701

3) Manufacturer

Wako Pure Chemicals Co.

4) Purity

97.8%

5) Storage condition

Test substance was stored in room temperature.

6) Handling precautions

Gloves, a mask, a head cap and a lab coat were worn when handling.

1.3 Vehicle

N,N-Dimethylformamide (DMF) was selected as a vehicle.

2 ANIMALS

2.1 Animal species

Female CBA/J mice, SPF (Charles River Japan, Inc)

2.2 Selection of animal species

CBA/J is a recommended mouse strain of OECD TG429. Additionally, it is confirmed as a high-sensitive strain in LLNA.

2.3 Age in weeks of animals at the start of the study

7 week-old

2.4 Age in weeks and body weights ranged of animals at applying of test substance

9 week-old, within ±20% of mean body weight

2.5 Procedure and period of quarantine and acclimation

Healthy animals in good general condition confirmed by a person in charge of animal management on arrival were quarantined 13 days. During the quarantine and acclimation period, clinical signs, body weights and excrement of the animals were monitored.

2.6 Grouping

Animals confirmed to be in good health with favorable body weight gains by a person in charge of animal management during the quarantine and acclimation period, were allocated to groups by random selection on the day before the start of applying test substance.

2.7 Identification

1) Animal

Animals were identified by marking the tail with colored marker.

2) Cage

Cages were identified by labels (Study code, Cage No., Animal No., Study Director, Study staff, species, strain, gender, sensitization period and test group).

3 HOUSING CONDITIONS

3.1 Housing condition

1) Animal room

Quarantine and acclimatization period

Air and humidity conditioned isolator (TAR-70MK6, Toyoriko Co Ltd) placed in the Animal room (#4107), CERI-Tokyo

Sensitization period

Air and humidity conditioned isolator (TAR-70MK6, Toyoriko Co Ltd) placed in the Animal room (#4107), CERI-Tokyo

2) Temperature

23°C (Actual temperature range was 21.3-24.6 °C.)

3) Relative humidity

55% (Actual relative humidity range was 50.0-64.0 %)

4) Air ventilation

50 cycles/h

5) Light-dark cycle

The rooms were artificially lighted for 12 h daily (7:00-19:00) and dark 12h (19:00-7:00)

6) Cage

Before grouping animals

Polycarbonate cage (280W×440D×205H mm)

After grouping animals

Polycarbonate cage (215W×320D×150H mm)

7) Density of animals in the Cage

Before grouping animals

Equal to or less than 10 animals per cage

After grouping animals

4 animals per cage

8) Frequency of changing equipments

On the day of grouping animals and after grouping animals, cage, fir chip and water bottle were changed once a week.

3.2 Food

1) Form

Pelleted diet (MF, ORIENTAL YEAST CO LTD)

2) Feeding

Free access via feeders

3) Lot No.

160419 A3

3.3 Water

1) Form

Water (chlorinated) from Sugito machi

2) Water supplying

Free access via water bottles

3.4 Fir chip

1) Form

White-Flakes (spruce wood chip, shaving by power planer)

2) Manufacturer

Charles River Japan, Inc

3) Lot No.

16.03.25

TEST METHOD

1 PRE-SCREEN TEST

1.1 Objective

Pre-screen test was performed in order to select the applicable maximum dose level in the main LLNA study, where the test substance induces neither excessive irritation nor systemic toxicity.

1.2 Grouping for pre-screen test

Group	Dose	Volume (μL/ear)	No. of application	N
PFHxA-NH4	0.5 %	25	once a day ×3days	2
	1 %	25		2
	2.5 %	25		2
	5 %	25		2
	10 %	25		2
	25 %	25		2
	50 %	25		2
	90 %	25		2

1.3 Preparations

1) Vehicle

DMF was used as a vehicle.

2) Time of preparation

Test solutions were stored in dark place until use.

1.4 Sensitization

1) Sensitization procedure

A 25μL of test solutions were applied to the dorsum of both ears of the mice using micro volume pipette.

2) Frequency of sensitization

Once a day for three days.

1.5 Observations and examination

1) General Condition

Clinical signs were observed at least once a day. Erythema scores of auricles were recorded individually according to the Scoring criteria of OECD TG429 adopted 2010.

Erythema Scores (OECD TG429, 2010)

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

2) Body Weights

Body weights were measured on the day of the first application (Day 1) and the day of final observation (Day 6).

3) Measurement of ear thickness

Ear thickness was measured in triplicate for each ear with the Digital micrometer (MDC-25MJ, Mitsutoyo) before application (Day 1), 48h after application (Day 3) and on the day of final observation (Day 6). The mean ear thickness was noted.

1.6 Dose selection

Dose elicits severe systemic toxicity, erythema excess score 3 or more than 25% increase of ear thickness compared with the data obtained before application would be excluded for test doses.

2 MAIN STUDY

2.1 Grouping

Group	Dose*	Volume (μL/ear)	Application	N female (Animal No.)
Vehicle treated control (DMF)	—	25	once per day ×3days	4 (V1 - V4)
PFHxA-NH4	25 %	25		4 (A1 - A4)
	50 %	25		4 (A5 - A8)
	90 %	25		4 (A9 - A12)
Positive control (HCA in DMF)	25%	25		4 (P1 - P4)

* Doses for test substance were decided according to the pre-screen test result.

2.2 Preparations

1) Vehicle

DMF was used as a vehicle.

2) Positive control substance

HCA (0.26 g) was accurately weighed and dissolved in DMF to 1 mL (25 w/v%) and stored by glass bottle.

3) Test substance

PFHxA-NH4 (0.9g) was accurately weighed and dissolved in DMF to 1 mL (90 w/v%). Additionally, 90 w/v% solution was diluted by DMF to prepare lower dose solutions (50 w/v% and 25 w/v%) .

4) ^{3}H-methyl thymidine

^{3}H-methyl thymidine (Moravek Biochemicals, Inc., 1 mCi/mL) was mixed with Phosphate-buffer saline (PBS) to make a 80 μCi/mL solution.

5) Time of preparation

All test solutions were prepared before sensitization and stored by shading glass bottle in a dark place. ^{3}H-methyl thymidine was prepared on the day of injection.

2.3 Sensitization

1) Sensitization procedure

A 25μL of test solutions were applied to the dorsum of both ears of the mice using micro volume pipette.

2) Time and frequency of sensitization

Once a day for three days.

2.4 ^{3}H-methyl thymidine injection

1) Administration route and method

A 0.25mL solution per mouse was injected using 29G Insulin Syringe (ss-05M2913, TERUMO CO. LTD.) via tail vein.

2) Time and frequency of injection

Once at 3 days after the final sensitization.

2.5 Observations and test

1) General Condition

Clinical signs were observed at least once a day.

2) Body Weights

Body weights were measured on the day of the first sensitization and the day of collection of lymph nodes.

3) Collection of lymph nodes and measurement of lymph node weight

Approximately 5h after administration of ^{3}H-methyl thymidine, the auricular lymph nodes were removed after euthanasia. The auricular lymph nodes were carefully dissected and trimmed of fascia and fat, and then weighed both sides of lymph nodes together.

4) Counting of incorporation of ^{3}H-methyl thymidine into auricular lymph nodes

The single-cell suspension of lymph node cell (LNC) was prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel mesh for generating a single-cell suspension. LNC were washed twice with an excess of PBS. Then, added 5% Trichloroacetic acid (TCA), then kept at 4°C for 18 hours. After 18 hours, pellets were resuspended in 1 mL TCA and transferred to 10 mL of scintilant (Ecolume^{TM}, MP Biomedicals), and incorporation of ^{3}H-methyl thymidine (DPM / mouse) was measured by liquid scintillation counter (Tri-Carb 3110TR、PerkinElmer).

2.6 Euthanasia

The animals used in this study were euthanized by cervical dislocation.

2.7 Handling of dead animal

No fatal cases of animals in this study.

2.8 Evaluation of the results

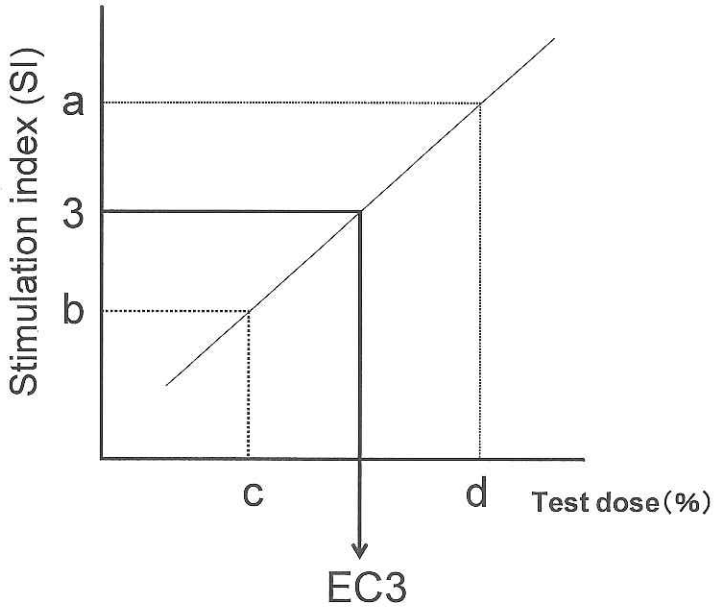
The mean incorporation of ^{3}H-methyl thymidine (DPM/ mouse) was calculated for the vehicle treated control group. Then, each value of incorporation of ^{3}H-methyl thymidine in all mice was divided by the mean DPM of the vehicle treated control group to calculate stimulation index (SI). SI of vehicle treated control and test substance group were expressed as means and standard errors. The decision process regards a result as positive when SI of test substance group ≥ 3.

$$SI = \frac{\text{Incorporation of } ^{3}\text{H-methyl thymidine of each animal}}{\text{Mean incorporation of } ^{3}\text{H-methyl thymidine of vehicle treated control}}$$

2.9 Calculation of EC3

EC3 value of test substance was calculated by the following equation, and the skin sensitization potency of test chemical was estimated by the criterion of Kimber I. et al (2003).

$$EC3=c+(3-b)(d-c)/(a-b)$$



The formula utilized the data points lying immediately above and below the SI value of 3 on the LLNA dose response curve containing the (SI, Test dose) coordinates (a, d) and (b, c), respectively.

Categorisation of contact allergens on the basis of relative skin sensitisation potency*

Category	EC3 (%)
Extreme	<0.1
Strong	≥0.1 to <1
Moderate	≥1 to <10
Weak	≥10 to ≤100

*Kimber I. et al., (2003)

2.10 GHS classification

Sub-category of test chemical was classified by the following criterion of GHS.

Sub-category 1A : EC3 value ≦2

Sub-category 1B : EC3 value ＞2

UNFORESEEN EVENTS THAT MAY HAVE AFFECTED THE RELIABILITY OF THE STUDY

No unforeseen events that might have affected the reliability of the study.

RESULTS

1 PRE-SCREEN TEST

1.1 General condition (Table 1)

No animals showed any abnormalities during prescreen test period.

1.2 Irritation (Table 1, Table 2)

No animals showed irritant reaction during prescreen test period.

1.3 Dose selection

The maximum dose level (90 %) was decided as the maximum concentration which was applicable to ear of mice without any serious toxic effects (systemic toxicity and/or severe local skin irritation), then lower 2 doses (50 % and 25 %) were added with serial dilution.

2 MAIN STUDY

2.1 General condition (Table 3)

No animals showed any abnormalities during test period.

2.2 Body weight (Table 3, Addendum 1)

No animals showed any abnormalities with regard to body weight change.

2.3 Lymph node weight (Table 4, Addendum 2, Figure 1)

2.3.1 Vehicle treated control group

The mean lymph node weight of vehicle treated control group with DMF was calculated as 4.5±0.4 mg, respectively.

2.3.2 Test substance group

The mean lymph node weight of 25 %, 50 % and 90 % test groups were calculated as 5.5±0.6 mg, 6.7±0.6 mg and 7.3±0.2 mg, respectively.

2.3.3 Positive control group

The mean lymph node weight was calculated as 9.3±0.3 mg.

2.4 Stimulation Index (SI) (Table 4, Addendum 3, Figure 2)

2.4.1 Vehicle treated control group

The mean SI of vehicle treated control group with DMF was calculated as 1.0±0.1.

2.4.2 Test substance group

The mean SI of 25 %, 50 % and 90 % test groups were calculated as 2.1±0.2, 3.3±0.6 and 4.1±0.5, respectively.

EC3 value was calculated as 43.3.

GHS classification was sub-category 1B.

2.4.3 Positive control group

The mean SI was calculated as 13.9±1.0.

DISCUSSION

Skin sensitization potential of PFHxA-NH4 was evaluated by Local Lymph Node Assay (LLNA). Study was conducted in accordance with the OECD TG429. Test solutions, 25%, 50%, and 90 % of PFHxA-NH4 was applied to the dorsum of both ears of female CBA/J mice in 9 weeks of age daily for three consecutive days, then ^{3}H-methyl thymidine was injected into all mice via the tail vein and incorporation of ^{3}H-methyl thymidine into auricular lymph node cells was measured. Stimulation Indices (SI) of 50 % of PFHxA-NH4 exceeded 3, this chemical was evaluated as positive in LLNA. Since EC3 of PFHxA-NH4 was 43.3, this chemical was classified as weak sensitizer. GHS sub-category of PFHxA-NH4 was classified 1B.
While, the known human contact allergens, α-Hexylcinnamaldehyde (HCA) used as positive control showed clear positive response with SI value of 13.9 (SI >3). Therefore it confirms the validity of this study.

REFERENCES

Kimber, I., Dearman, R.J., Scholes, E.W., Basketter, D.A. (1994). The local lymph node assay: developments and applications, Toxicology, 93, 13-31.

Organization for Economic Corporation and Development (OECD, 2010). Skin Sensitisation: Local Lymph Node Assay, TG-429 (Adopted: 22nd July 2010).

Kimber I., Basketter D. A., Butler M., Gamer A., Gerberick G. F., Newsome C., Steiling W., and Vohr H.-W., (2003) Classification of contact allergens according to potency: proposals. Food and Chemical Toxicology, 41: 1799 – 1809.

Globally Harmonized System of Classification and Labelling of Chemicals (GHS) 6th revised edition (United Nations, 2015). CHAPTER 3.4 RESPIRATORY OR SKIN SENSITIZATION.

Table 1 Body weights and general condition (Pre-screen test)

Group	% tested	N	Body weights (g)				clinical signs	Erythema Scores
			day 1		day 6			
			Mean	SE	Mean	SE	day 1 - 6	day 1 - 6
PFHxA-NH4	0.5%	2	20.7	1.08	20.6	0.74	NAD	0
	1%	2	20.9	0.41	19.8	0.94	NAD	0
	2.5%	2	20.5	0.41	20.0	0.21	NAD	0
	5%	2	19.0	0.21	19.4	0.09	NAD	0
	10%	2	19.9	0.42	20.2	0.28	NAD	0
	25%	2	20.2	1.19	21.8	1.56	NAD	0
	50%	2	20.7	0.92	22.4	0.85	NAD	0
	90%	2	20.0	1.07	20.3	1.38	NAD	0

N: number of animals

SE: standard error

NAD: no abnormalities detected during Observation period

Table 2 Ear thickness (Pre-screen test)

Group	% tested	N	Ear thickness (μm)									
			day 1		day 3				day 6			
			Mean (L)	Mean (R)	Mean (L)		Mean (R)		Mean (L)		Mean (R)	
PFHxA-NH4	0.5%	2	225.7	230.7	240.7	106.6%	239.0	103.6%	238.2	105.5%	240.5	104.3%
	1%	2	228.8	227.8	241.0	105.3%	236.8	104.0%	241.0	105.3%	237.0	104.0%
	2.5%	2	229.7	235.0	237.7	103.5%	239.7	102.0%	242.3	105.5%	239.2	101.8%
	5%	2	226.5	224.8	239.0	105.5%	235.8	104.9%	240.8	106.3%	241.7	107.5%
	10%	2	233.0	230.5	237.7	102.0%	239.7	104.0%	241.3	103.6%	244.5	106.1%
	25%	2	227.2	228.8	242.2	106.6%	242.3	105.9%	242.0	106.5%	239.7	104.7%
	50%	2	233.0	225.5	244.5	104.9%	246.2	109.2%	238.7	102.4%	243.5	108.0%
	90%	2	228.2	232.5	234.2	102.6%	236.3	101.6%	241.3	105.8%	242.8	104.4%

N: number of animals

L: left ear, R: right ear

Table 3 Body weights and general condition

Group	% tested	N	Body weights (g)				clinical signs
			day 1		day 6		
			Mean	SE	Mean	SE	day 1 - 6
Vehicle treated control (DMF)	-	4	21.9	0.8	20.8	0.9	NAD
PFHxA-NH4	25 %	4	22.1	0.7	21.4	0.5	NAD
	50 %	4	22.4	0.4	22.3	0.5	NAD
	90 %	4	22.8	0.7	23.2	0.6	NAD
Positive control (HCA in DMF)	25 %	4	22.8	0.4	22.2	0.5	NAD

N: number of animals

SE: standard error

DMF: *N,N*-Dimethylformamide

HCA: α-Hexylcinnamaldehyde

NAD: no abnormalities detected during Observation period

Table 4 Summary of the results in standard local lymph node assay

Group	% tested	N	Lymph node weight (mg)		DPM		Stimulation index (SI)		EC3(%)
			Mean	SE	Mean	SE	Mean	SE	
Vehicle treated control (DMF)	-	4	4.5	0.4	426	50	1.0	0.1	-
PFHxA-NH4	25 %	4	5.5	0.6	912	82	2.1	0.2	43.3
	50 %	4	6.7	0.6	1410	245	3.3	0.6	
	90 %	4	7.3	0.2	1762	197	4.1	0.5	
Positive control (HCA in DMF)	25 %	4	9.3	0.3	5901	436	13.9	1.0	-

N: number of animals

SE: standard error

DPM: Disintegration per minute

DMF: *N,N*-Dimethylformamide

HCA: α-Hexylcinnamaldehyde

Addendum 1 Body weights of individual animals (g)

Group		Animal No.	day 1 BW (g)	day 6 BW (g)
Vehicle treated control (DMF)	-	V1	22.77	22.54
		V2	22.46	21.87
		V3	19.62	18.24
		V4	22.64	20.60
PFHxA-NH4	25 %	A1	21.19	20.76
		A2	20.64	20.37
		A3	22.89	21.87
		A4	23.69	22.43
	50 %	A5	21.94	21.22
		A6	23.17	22.48
		A7	21.40	21.73
		A8	22.99	23.66
	90 %	A9	22.99	23.50
		A10	21.94	22.36
		A11	21.71	22.33
		A12	24.64	24.73
Positive control (HCA in DMF)	25 %	P1	22.42	21.92
		P2	23.03	22.07
		P3	21.99	21.22
		P4	23.79	23.52

DMF: *N,N*-Dimethylformamide

HCA: α-Hexylcinnamaldehyde

Addendum 2 Lymph node weights of individual animals (mg)

Group		Animal No.	Lymph node weight (mg)
Vehicle treated control (DMF)	-	V1	4.3
		V2	5.3
		V3	3.5
		V4	4.9
PFHxA-NH4	25 %	A1	5.4
		A2	3.9
		A3	5.9
		A4	6.8
	50 %	A5	5.8
		A6	7.8
		A7	5.5
		A8	7.8
	90 %	A9	7.6
		A10	7.7
		A11	6.8
		A12	7.2
Positive control (HCA in DMF)	25 %	P1	8.5
		P2	10.1
		P3	9.3
		P4	9.2

DMF: *N,N*-Dimethylformamide

HCA: α-Hexylcinnamaldehyde

Addendum 3 DPM and SI values of individual animals

Group		Animal No.	DPM	Stimulation index (SI)
Vehicle treated control (DMF)	-	V1	533	1.3
		V2	334	0.8
		V3	490	1.1
		V4	348	0.8
PFHxA-NH4	25 %	A1	878	2.1
		A2	928	2.2
		A3	1120	2.6
		A4	724	1.7
	50 %	A5	1376	3.2
		A6	946	2.2
		A7	1224	2.9
		A8	2096	4.9
	90 %	A9	1983	4.7
		A10	2182	5.1
		A11	1308	3.1
		A12	1577	3.7
Positive control (HCA in DMF)	25 %	P1	5300	12.4
		P2	5444	12.8
		P3	7189	16.9
		P4	5671	13.3

DPM: Disintegration per minute

DMF: *N,N*-Dimethylformamide

HCA: α-Hexylcinnamaldehyde

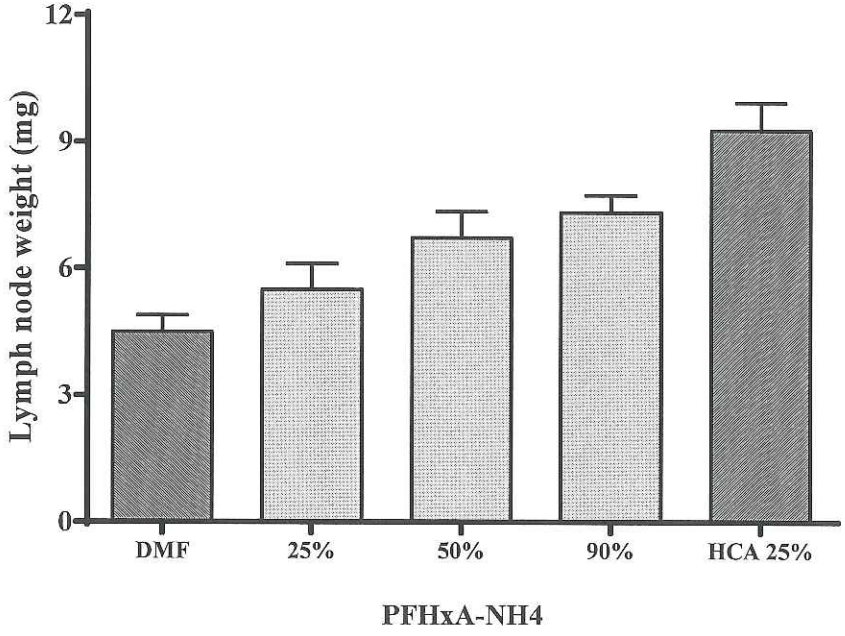


Figure 1 Lymph node weights in the local lymph node assay

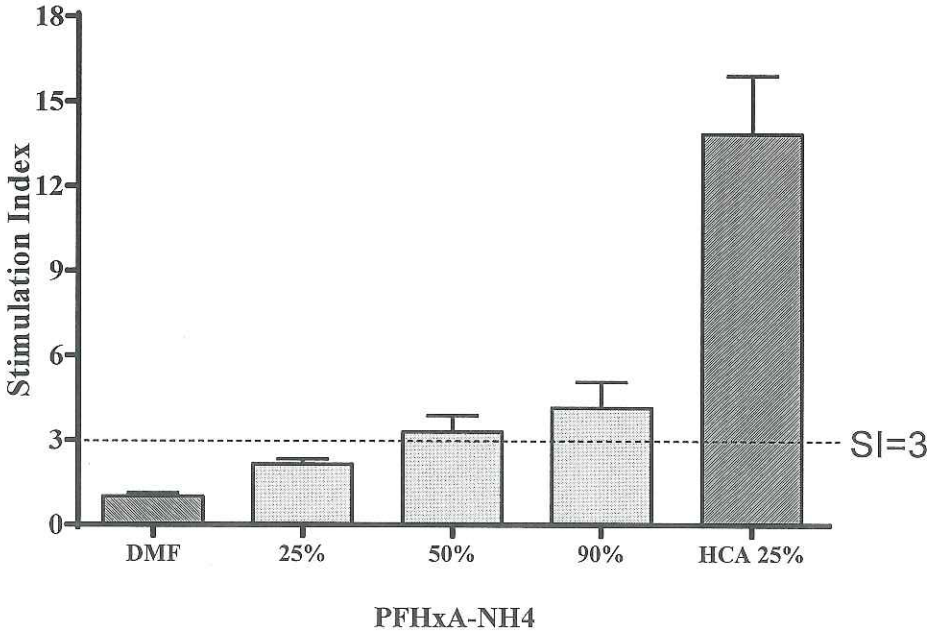


Figure 2 Stimulation indices in the local lymph node assay

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June 1, 2017

Date

Chemicals Assessment and Research Center
Chemicals Evaluation and Research Institute, Japan