Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate; tetrakis(2,6-dimethylphenyl) 1,3-phenylene bis(phosphate)

EC Number: 432-770-2

CAS Number: 139189-30-3

Index Number: 015-192-00-1

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CLH report prepared by CS Regulatory Ltd. in accordance with Article 37(6) of CLP

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CONTENTS

1 PHYSICAL HAZARDS

1.1 Explosives

1.1.1 [Study 1]

Study 1 reference:

Tremain, S.P. 1999

Test type

EU Method A.14 (Explosive properties)

Detailed study summary and results:

Test material
Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Material and methods

Not specified.

Results

Evaluation of results: non explosive

Study results:
Small-scale preliminary tests:
More sensitive to shock than m-dinitrobenzene - migrated information: (not measured/tested)
More sensitive to friction than m-dinitrobenzene - migrated information: (not measured/tested)
Explosive under influence of flame - migrated information: (not measured/tested)
Explosive (not specified) - migrated information: (negative (not further specified))
Remarks:

BAM fall hammer test

Test	Observations
1	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.
2	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.
3	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.
4	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.

5	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.
6	There were no signs of ignition or explosion on impact. When the test assembly was dismantled, there were no signs of decomposition.

BAM friction test

Test	Observations
1	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.
2	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.
3	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.
4	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.
5	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.
6	There were no signs of ignition or explosion. A black mark on the porcelain plate and peg indicated decomposition.

Koenen steel tube test

6 mm orifice plate

Test	Time (s)	Observations
1	119	An orange/blue flame from the orifice.
	149	The orange/blue flame turned to an orange flame and increased in size.
	162	Black smoke was emitted.
	173	Burning test material was ejected from the orifice.
	209	A dome of charred test material was formed around the orifice.
	222	The orange flame decreased in size.
	300	The test was stopped. The orange flame remained. The tube was recovered intact.
2	113	An orange/blue flame from the orifice.
	142	The orange/blue flame turned to an orange flame and increased in size.
	159	Black smoke was emitted.
	168	Burning test material was ejected from the orifice.
	204	A dome of charred test material was formed around the orifice.
	218	The orange flame decreased in size.

	300	The test was stopped. The orange flame remained. The tube was recovered intact.
3	114	An orange/blue flame from the orifice.
	140	The orange/blue flame turned to an orange flame and increased in size.
	161	Black smoke was emitted.
	172	Burning test material was ejected from the orifice.
	203	A dome of charred test material was formed around the orifice.
	215	The orange flame decreased in size.
	300	The test was stopped. The orange flame remained. The tube was recovered intact.

2 mm Orifice Plate

Test	Time (s)	Observations
1	101	An orange/blue flame from the orifice.
	130	The orange/blue flame turned to an orange flame and increased in size.
	151	Black smoke was emitted.
	162	Burning test material was ejected from the orifice.
	186	The orange flame turned to a jet of orange flame.
	195	The jet of orange flame reverted to an orange flame and decreased in size. A dome of charred test material was formed around the orifice.
	300	The test was stopped. The orange flame remained. The tube was recovered intact.
2	96	An orange/blue flame from the orifice.
	124	The orange/blue flame turned to an orange flame and increased in size.
	150	Black smoke was emitted.
	157	Burning test material was ejected from the orifice.
	181	The orange flame turned to a jet of orange flame.
	201	The jet of orange flame reverted to an orange flame and decreased in size. A dome of charred test material was formed around the orifice.
	300	The test was stopped. The orange flame remained. The tube was recovered intact.
3	104	An orange/blue flame from the orifice.
	128	The orange/blue flame turned to an orange flame and increased in size.
	147	Black smoke was emitted.

	158	Burning test material was ejected from the orifice.
	178	The orange flame turned to a jet of orange flame.
	194	The jet of orange flame reverted to an orange flame and decreased in size. A dome of charred test material was formed around the orifice.
	300	The test was stopped. The orange flame remained. The tube was recovered intact.

1.2 Flammable gases (including chemically unstable gases)

Not applicable – test substance is a solid.

1.3 Oxidising gases

Not applicable – test substance is a solid.

1.4 Gases under pressure

Not applicable – test substance is a solid.

1.5 Flammable liquid

Not applicable – test substance is a solid.

1.6 Flammable solids

1.6.1 [Study 1]

Study 1 reference:

Tremain, S.P. 1999

Test type

Flammable solids according to EU Method A.10 (Flammability (Solids)).

Detailed study summary and results:

Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Material and methods

Not specified.

Results

Evaluation of results: non flammable
Study results:
Pyrophoric solids:
ignition time on contact with air - Ignition on contact with air refers to either Pyrophoric solids or Pyrophoric liquids depending on physical state (migrated information). (no ignition on contact with air (not further specified) - corresponding to 'no' in migrated source record.)
Pyrophoric liquid:
ignition time on contact with air - Ignition on contact with air refers to either Pyrophoric solids or Pyrophoric liquids depending on physical state (migrated information). (no ignition on contact with air (not further specified) - corresponding to 'no' in migrated source record.)
Self-heating substances/mixtures:
Substances/ mixture which in contact with water emit flammable gases:
Remarks:
The pile failed to ignite during the two minutes that the Bunsen flame was applied.
The result of the preliminary screening test obviated the need to perform the main test.
Moisture content

	Determination A	Determination B
a) Mass of loss bottle (g)	20.6951	20.1423
b) Mass of loss bottle and test material (g)	21.6930	21.1396
c) Mass of loss bottle and test material after drying (g)	21.6930	21.1396
Moisture content (% w/w)	<0.1	<0.1
Mean moisture content (% w/w)	<0.1	<0.1

1.7 Self-reactive substances

No data are available.

1.8 Pyrophoric liquids

Not applicable – test substance is a solid.

1.9 Pyrophoric solid

1.9.1 [Study 1]

Study 1 reference:

Tremain, S.P. 1999

Test type

Flammable solids according to EU Method A.10 (Flammability (Solids)).

Detailed study summary and results:

Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Material and methods

Not specified.

Results

Evaluation of results: non flammable
Study results:
Pyrophoric solids:
ignition time on contact with air - Ignition on contact with air refers to either Pyrophoric solids or Pyrophoric liquids depending on physical state (migrated information). (no ignition on contact with air (not further specified) - corresponding to 'no' in migrated source record.)
Pyrophoric liquid:
ignition time on contact with air - Ignition on contact with air refers to either Pyrophoric solids or Pyrophoric liquids depending on physical state (migrated information). (no ignition on contact with air (not further specified) - corresponding to 'no' in migrated source record.)
Self-heating substances/mixtures:
Substances/ mixture which in contact with water emit flammable gases:
Remarks:
The pile failed to ignite during the two minutes that the Bunsen flame was applied.
The result of the preliminary screening test obviated the need to perform the main test.
Moisture content

	Determination A	Determination B
a) Mass of loss bottle (g)	20.6951	20.1423
b) Mass of loss bottle and test material (g)	21.6930	21.1396
c) Mass of loss bottle and test material after drying (g)	21.6930	21.1396
Moisture content (% w/w)	<0.1	<0.1
Mean moisture content (% w/w)	<0.1	<0.1

1.10 Self-heating substances

No data are available.

1.11 Substances which in contact with water emit flammable gases

No data are available.

1.12 Oxidising liquids

Not applicable – substance is a solid.

1.13 Oxidising solids

1.13.1 [Study 1]

Study 1 reference:

Tremain, S.P. 1999

Test type

Oxidising solids
Contact with: powdered cellulose, according to EU Method A.17 (Oxidising Properties (Solids))

Detailed study summary and results:

Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2

Material and methods

Not specified.

Results

Evaluation of results: no oxidising properties - Migrated information
Test results:
Oxidising solids:
test mixture (not specified) - migrated information: maximum burning rate: 0 (The test material/cellulose mixtures failed to ignite.)
reference mixture (not specified) - migrated information: maximum burning rate: 1.361 mm/s (migrated information) (Mixture = 70% barium nitrate, 30% cellulose)
Remarks:
Preliminary test
The pile ignited, but self-extinguished 1 second after the Bunsen flame was removed.
Main test
The test material/cellulose mixtures failed to propagate combustion.

1.14 Organic peroxides

No data are available.

1.15 Corrosive to metals

No data are available.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1.1 [Study 1]

Study 1 reference:

NONS written assessment; updated 2012.

Test type

No test method followed. Written assessment based on available Q1 GLP compliant data.

Test substance

Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Detailed study summary and results:

The substance is an aromatic organo-phosphorus ester of molecular weight that does not preclude absorption. No specific predictions about toxicokinetic behaviour can be made from the chemical structure. The structure suggests potential for cholinesterase inhibition, but this was specifically investigated in a repeated dose oral toxicity study with no effect identified. The substance is a non-volatile powder of non-respirable particle size, so inhalation exposure is not anticipated. Non-enzymatic hydrolysis is unlikely so exposure to degradants is not applicable.

Absorption:
Acute oral and dermal toxicity studies showed no treatment related effects and therefore provide no evidence of absorption by either route. In a repeated dose oral toxicity study also there is no significant evidence of adsorption. The substance has very high log Pow value, which would suggest ready diffusion across membranes and hence absorption. In view of the extremely low water solubility, however, this may not be a true representation of lipophilicity. Exposure in a screening reproductive toxicity test indicates no effects to parent or F1 animals and no adverse effects were indicated by mutagenicity testing.

Distribution:
There is no experimental evidence to indicate distribution except, perhaps, to the liver in the repeated dose oral toxicity study. The extremely high Pow values obtained by testing and QSAR may be suggestive of potential for accumulation, but bioaccumulation potential tends to decrease as Pow becomes increasingly high, becoming more an effect of low water solubility rather than accumulation. This observation is further borne out by the data available from fish bioaccumulation and QSAR estimations of BCF.

Metabolism:
The studies conducted provide no information about potential metabolism, but from the chemical structure, biotransformation of any absorbed substance would be expected. Ester hydrolysis by hydrolase enzymes could occur together with oxidative metabolism by the microsomal mixed function oxidase system and subsequent conjugation reactions.

Excretion:
There is no experimental evidence to indicate a route of excretion but the parent substance is not sufficiently water-soluble for elimination in its unchanged form in urine or bile, but may be eliminated in faecal matter. Biotransformation of any absorbed substance is, however, anticipated and the resulting metabolites could be

eliminated either in urine, bile or faeces. As the parent substance non-volatile and could not be eliminated via the lungs in expired air.

3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

Not relevant for this CLH proposal.

3.2 Acute toxicity - dermal route

Not relevant for this CLH proposal.

3.3 Acute toxicity - inhalation route

Not relevant for this CLH proposal.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 [Study 1]

Study reference:

Anonymous, 1995

Detailed study summary and results:

A study was performed for the purposes of EU NONS notification to assess the irritancy potential of the test material to the skin of the New Zealand White rabbit. The method used was OECD Guideline No. 404. A single 4 -hour, semi-occluded application of the test material to the intact skin of three rabbits produced very slight erythema. All treated skin sites appeared normal at the 24 -hour observation.

Test type

According to OECD Guideline 404 (Acute Dermal Irritation / Corrosion) ; according to EU Method B.4 (Acute Toxicity: Dermal Irritation / Corrosion)

Test substance

Test material
Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Species

Rabbit common species

Strain
New Zealand White rabbit

Administration/exposure

Type of coverage: semiocclusive
Preparation of test site: other: clipped free of fur
Vehicle: other: moistened with distilled water
Controls: no
Amount / concentration applied: TEST MATERIAL- Amount(s) applied (volume or weight with unit): 0.5 g was moistened with 0.5 ml of distilled water
Concentration (if solution): not applicable
VEHICLE: not applicable
Duration of treatment / exposure: 4 hours
Observation period: 72 hours
Number of animals: 3

Details on study design

TEST SITE: Area of exposure: 2.5 cm x 2.5 cm
% coverage: not stated
Type of wrap if used: surgical adhesive tape (BLENDERM: approximate sixe 2.5 cm x 4 cm)
REMOVAL OF TEST SUBSTANCE- Washing (if done): removed by gentle swabbing with cotton wool soaked in distilled water
Time after start of exposure: 4 hours
SCORING SYSTEM: The score for erythema and oedema at the 24 and 72 hour readings were totalled for the 3 test rabbits (12 values) and this total was divided by 6 to give the primary irritation index of the test material.The EU classification system was used.

Results and discussion

Irritation parameter primary dermal irritation index (PDII) **Basis** mean **Time point** other: calculated at 24, 48 and 72 hours. **Score** 0.0 **Reversibility** fully reversible
Irritation parameter erythema score 108 Female **Basis** mean **Time point** other: mean of 24, 48 and 72 hours **Score** 0.0 **Max. score** 0.0

Reversibility fully reversible
Irritation parameter erythema score 114 Female **Basis** mean **Time point** other: mean score for 24, 48 and 72 hours **Score** 0.0 **Max. score** 0.0 **Reversibility** fully reversible
Irritation parameter erythema score 91 Female **Basis** mean **Time point** other: mean score for 24, 48 and 72 hours **Score** 0.0 **Max. score** 0.0 **Reversibility** fully reversible
Irritation parameter edema score 108 Female **Basis** mean **Time point** other: mean score for 24, 48 and 72 hours **Score** 0.0 **Max. score** 0.0 **Reversibility** fully reversible
Irritation parameter edema score 114 Female **Basis** mean **Time point** other: mean score for 24, 48 and 72 hours **Score** 0.0 **Max. score** 0.0 **Reversibility** fully reversible
Irritation parameter edema score 91 Female

Basis
mean
Time point
other: mean score for 24, 48 and 72 hours
Score
0.0
Max. score
0.0
Reversibility
fully reversible

Very slight erythema was noted at 2 treated skin sites one hour after patch removal.
All treated skin sites appeared normal at the 24 hour observation period.

Interpretation of results
not irritating Migrated information Criteria used for interpretation of results: EU

Conclusions
The test material, PX-200, produced a primary irritation index of 0.0 and was classified as a non irritant to rabbit skin according to the Draize classification scheme. No corrosive effects were not ed.The test material did not meet the criteria for classification as irritant or corrosive according to EU labelling regulations. No symbol and risk phrase are required.

3.4.2 Human data

3.4.2.1 [Study 1]

Study reference:

Yukio Yanagimoto 2002

Detailed study summary and results:

A human patch volunteer study was conducted for the purposes of worker safety assessment under the supervision of a medical practitioner and according to a Japanese dermatological standard.

Coverage: occlusive
Vehicle: unchanged (no vehicle)
Taro Kawamura, et. al, Basic Study for Standardisation of patch test, Japanese Dermatological Association News, 80, 301-314 (1970). Test volunteers are exposed and initial pain responses recorded. Following exposure, volunteers are exposed to the test substance for a continual period of 48 hours with occluded dressing and results reviewed at the end of the 48 hour exposure period according to the standard of Japan Patch Test Association (Japanese standard).

Type of coverage: occlusive
Vehicle: unchanged (no vehicle)
Controls: yes, concurrent no treatment

Observation period: 48 hours
Number of volunteers: 20

With 20 healthy volunteers as subjects, 0.1g of the test substance was applied on the circular cloth area of adhesive tape for patch test. The adhesive tape was applied on the inward upper arms of the subjects. As control, adhesive tape only was applied in parallel with the test substance on the inner upper arms. The skin symptoms of contact area and control area were macroscopically observed and evaluated at 48 hours after the application of the test substance, and pictures of them were taken.
A little amount of vaselline was applied on the cloth area in order to adhere the test substance to circular cloth area of the adhesive tape.

Furthermore, the subjects were asked to submit a letter of acceptance and precautions were provided to them before the test was conducted.

Presence or absence of irritation symptoms (erhythema, oedema, blister) was assessed for findings on the skin where the test substance contacted according to the standard of Japan Patch Test Association (Japanese standard)
The standard of Japan Patch Test Association (Japanese standard)1)

0	(-)	No reaction
0.5	(±)	Slight erythema
1	(+)	Erythema
2	(++)	Erythema + oedema
3	(+++)	Erythema + oedema + papule, serosanguineous papule, vesicular
4	(++++)	Large blister

Results

Irritation parameter
overall irritation score
Basis
mean
Time point
other: 48 hours
Score
0.0
Max. score
0.0
Reversibility
other: no results observed

Not irritating - Migrated information Criteria used for interpretation of results: other: Taro Kawamura, et. al, Basic Study for Standardisation of patch test, Japanese Dermatologial Association News, 80, 301-314 (1970)
overall irritation score
0 of max. 0 (Time point: 48 hours) Reversibility: no results observed

Volunteer No. Picture No.	Person subject to the test (sex, age)	Score	
		Test substance	Control

					(Blank)
1	*Name*	M	24	0	0
2	*Name*	M	19	0	0
3	*Name*	M	29	0	0
4	*Name*	M	25	0	0
5	*Name*	M	28	0	0
6	*Name*	M	21	0	0
7	*Name*	M	26	0	0
8	*Name*	F	20	0	0
9	*Name*	M	19	0	0
10	*Name*	M	31	0	0
11	*Name*	M	27	0	0
12	*Name*	M	27	0	0
13	*Name*	M	25	0	0
14	*Name*	M	20	0	0
15	*Name*	M	22	0	0
16	*Name*	M	27	0	0
17	*Name*	M	20	0	0
18	*Name*	M	22	0	0
19	*Name*	M	31	0	0
20	*Name*	M	21	0	0

Interpretation of results
not irritating Migrated informationCriteria used for interpretation of results: other: Taro Kawamura, et. al, Basic Study for Standardisation of patch test, Japanese Dermatologial Association News, 80, 301-314 (1970)
Conclusions
The substance is not irritant to human skin

3.4.3 Other data

No other data available.

3.5 Serious eye damage/eye irritation

Not relevant for this CLH proposal.

3.6 Respiratory sensitisation

Not relevant for this CLH proposal.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 [Study 1]

Study reference:

Anonymous (1999)

Detailed study summary and results:

Test type

Skin sensitisation: in vivo (non-LLNA)
according to OECD Guideline 406 (Skin Sensitisation); according to EU Method B.6 (Skin Sensitisation); GPMT

GLP compliance
yes (incl. certificate) UK GLP Standards (Schedule 1, Good Laboratory Practice Regulations 1997 (SI 1997/654)).

Test substance

Test material: Tetrakis (2,6-dimethylphenyl)-m-phenylene biphosphate; Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2 (see Annex 2 for more information), Form:

Species: guinea pig
Strain: Dunkin-Hartley guinea pig
Sex: female

Administration/exposure

Induction

Route intradermal **Vehicle** arachis oil
Concentration / amount 75%, 50%, 25% and 10% w/w in Arachis oil BP.

Challenge

Route epicutaneous, occlusive **Vehicle** arachis oil
Concentration / amount

75%, 50%, 25% and 10% w/w in Arachis oil BP.

No. of animals per dose
15

Details on study design
RANGE FINDING TESTS: The concentration of test material to be used at each stage of the main study were determined by sighting tests in which groups of guinea pigs were treated with various concentrations of the test material.

MAIN STUDY:

INDUCTION EXPOSURE
- No. of exposures: 3
- Exposure period: not applicable as injections
- Test groups: 10 guinea pigs used for the main study
- Control group: 5 guines pigs used for the control
- Site: shoulder region approximately 40 mm x 60mm
- Frequency of applications: not stated
- Duration: 24 to 28 hours
- Concentrations: 75%, 50%, 25
% and 10% w/w in Arachis oil BP.B.

CHALLENGE EXPOSURE
- No. of exposures: 1
- Day(s) of challenge: Day 7
- Exposure period: 48 hours
- Test groups: 10 guinea pigs
- Control group: 5 guinea pigs
were used for the control group
- Site: shoulder region
- Concentrations: 75% w/w in Arachis oil BP
Evaluation
- (hr after challenge): 48 hours

Two main phases were involved in the main study; (a) an induction of a response and (b) a challenge of that response.

Induction:

Induction of the test animals: Shortly before treatment on Day 0 the hair was removed from an area approximately 40 mm x 60 mm on the shoulder region of each animal with vetinary clippers. A row of three (0.1 ml each) was made on each side of the mid-line.

The injections were:
a). Freund's Complete Adjuvant plus distilled water in the ratio 1:1
b). A 5% w/v formulation of the test material in Arachis oil BP
c). A 5% w/v formulation of the tets material in a 1:1 preparation of Freund's Complete Adjuvant plus distilled water.

Approximately 24 and 48 hours after intradermal injection the degree of erythema at the test material injection sites were evaluated.

One week later (Day 7) the same area on the shoulder region was clipped again and treated with a topical application of the test material formulation. A filter paper patch (WHATMAN No4: approximate size 40 mm x 20 mm), loaded with the tets material formulation (75% w/v in Arachis oil BP) as a thick, even layer was applied to the prepared skin and held in place with a strip of surgical adhesive tape covered with an overlapping length of aluminium foil. The patch and foil were further secured with a strip of elastic adhesive bandage wound in a double layer around the torso of each animal. This occlusive dressing was kept in place for 48 hours.

The degree of erythema and oedema was calculated 1 and 24 hours after the removal of the patches.

Induction of the control animals:
Intradermal injections were administered using an identical procedure to that used for the test animals except the injections were:
a). Freund's Complete Adjuvant plus distilled water in the ration 1 : 1
b). arachis oil BP
c). 50% w/v formulation of arachis oil BP in a 1 : 1 mixture of Freund's Complete Adjuvant/distilled water.

Challenge:
Shortly before treatment on Day 21, an area of approximately 50 mm x 70 mm on both flanks of each animal, was clipped free of hair with vetinary clippers. A square filter paper patch (WHATMAN No4: approximate size 20 mm x 20 mm), loaded with a thick, even layer of test material at the maximum non-irritant concentration (75% w/w in Arachis oil BP) was applied to the shorn right flank of each animal and held with surgical tape. To ensure that the maximum non-irritant concentration was used at challenge, the test material at a concentration of 50% w/w in arachis oil BP was similarly applied to a skin site on the left shorn flank. The patches were occluded with an overlapping length of aluminium foil and secured with a strip of elastic adhesive bandage wound in a double layer around the torso of each animal.

After 24 hours the dressing was carefully cut using blunt-tipped scissors, removed and discarded. The challenge sites were swabbed with cotton wool and soaked in diethyl ether to remove the residual material. The position of the treatment sites was identified by using a black indelible marker-pen.

Interpretation of results:
The material will be classified as sensitising and assigned the symbol "Xi", the indication of danger irritant and the risk phrase R 43 may cause sensitisation by skin contact if 30% or more of the test animals show a sensitisation reponse.

Results and discussion

Key result
false
Reading
1st reading
Hours after challenge
24.0
Group
test group
Dose level
75% test material in arachis oil BP
No. with + reactions
3
Total no. in group
10
Clinical observations
2 animals showed signs of just eythem and 1 animal showed signs of both erythema and oedema

Remarks on result
other: see Remark Reading: 1st reading. . Hours after challenge: 24.0. Group: test group. Dose level : 75% test material in arachis oil BP. No with. + reactions: 3.0. Total no. in groups: 10.0. Clinical observations: 2 animals showed signs of just eythem and 1 animal showed signs of both erythema and oedema.

Key result
false
Reading
2nd reading
Hours after challenge
48.0
Group
test group
Dose level
75% test material in arachis oil BP
No. with + reactions
2
Total no. in group
10
Clinical observations
2 animals have shown mild signs of erythema
Remarks on result
other: Reading: 2nd reading. . Hours after challenge: 48.0. Group: test group. Dose level: 75% test material in arachis oil BP. No with. + reactions: 2.0. Total no. in groups: 10.0. Clinical observations: 2 animals have shown mild signs of erythema.

Key result
false
Reading
1st reading
Hours after challenge
24.0
Group
test group
Dose level
50% test material in arachis oil
No. with + reactions
4
Total no. in group
10
Clinical observations
3 animals have shown signs of just erythema and 1 animal has shown signs of erythema and oedema
Remarks on result
other: see Remark Reading: 1st reading. . Hours after challenge: 24.0. Group: test group. Dose level: 50% test material in arachis oil. No with. + reactions: 4.0. Total no. in groups: 10.0. Clinical observations: 3 animals have shown signs of just erythema and 1 aimal has shown signs of erythema and oedema.

Key result
false
Reading
2nd reading
Hours after challenge
48.0

Group
test group
Dose level
50% test material in arachis oil
No. with + reactions
3
Total no. in group
10
Clinical observations
3 animals have shown signs of erythema
Remarks on result
other: Reading: 2nd reading. . Hours after challenge: 48.0. Group: test group. Dose level: 50% test material in arachis oil. No with. + reactions: 3.0. Total no. in groups: 10.0. Clinical observations: 3 animals have shown signs of erythema.

Key result
false
Reading
1st reading
Hours after challenge
1.0
Group
positive control
No. with + reactions
2
Total no. in group
5
Clinical observations
2 animals showed signs of erythema
Remarks on result
other: Reading: 1st reading. . Hours after challenge: 1.0. Group: positive control. No with. + react ions: 2.0. Total no. in groups: 5.0. Clinical observations: 2 animals showed signs of erythema .

Key result
false
Reading
2nd reading
Hours after challenge
24.0
Group
positive control
No. with + reactions
0
Total no. in group
5
Clinical observations
no effects were noted
Remarks on result
other: Reading: 2nd reading. . Hours after challenge: 24.0. Group: positive control. No with. + reac tions: 0.0. Total no. in groups: 5.0. Clinical observations: no effects were noted.

Main study:
Discrete or patchy to intense erythema and swelling was noted at the intradermal induction sites of all test group animals at the 24 hour observation with discrete or patchy to moderate and confluent

erythema at the 48 hour observation period.

Discrete or patchy to moderate and confluent erythema was noted at the intradermal induction sites of all control group animals at the 24 hour observation period with discrete or patchy erythema at the 48 hour observation period.

Skin Reactions:
Discrete or patchy erythema was noted at the topical induction sites of six test group animals at the 1 - hour observation period. No evidence of erythema or oedema was noted after this period. Also discrete or patchy erythema was noted at the topical induction sites of two control group animals at the 1 hour observation. No evidence of erythema or oedema was noted at the topical induction sites of the control group animals at the 24 hour observation period.

Results observed after the topical challenge:

75% w/w in Arachis oil BP
Positive skin responses (discrete or patchy erythema with or without very slight oedema) were noted at the challenge sites of three test group animals at the 24 hour observation period and two test group animals at the 48 -hour observation period.
No skin reactions were noted at the challenge sites of the control group animals at the 24 and 48 hour observation.
50% w/w in Arachis Oil BP
Positive skin responses (discrete or patchy to moderate and confluent erythema with or without very slight oedema) were noted at the challenge sites of four test group animals at the 24 observation and three test group animals at the 48 hour observation.
Body weight gains of guinea pigs in the test group, between Day 0 and Day 24, were comparable to those observed in the control group animals over the same period.

Interpretation of results
Sensitising

Conclusions
The test material was classified as a sensitiser according to EU labelling regulations.

3.7.1.2 [Study 2]

Study reference:
Anonymous (2017)

Detailed study summary and results:

Test type
Skin sensitisation: in vivo (LLNA)
according to OECD Guideline 442B (Skin Sensitisation: Local Lymph Node Assay: BrdU-ELISA)

Test substance
- Tetrakis (2,6-dimethylphenyl) m-phenylene bisphosphate, Form: solid: particulate/powder

Species
mouse
Strain
CBA:J mouse

Sex
female

Vehicle
acetone/olive oil (4:1 v/v)

Concentration
The test substance was dissolved in AOO, which is a recommended vehicle in the OECD TG 442B, at a concentration of 50.0 w/v%. In addition, the solution was visually stable at room temperature for four hours after preparation. Therefore, the AOO was selected as a vehicle.

No. of animals per dose
Pre-screen test: 5 animals
Main study: 4 animals per dose group

Details on study design

PRE-SCREEN TEST
Objective: The objective of the pre-screen test was to set the dosage for the main study.
Grouping: Five dose levels of the test substance were set as follow. Since the test substance was solid, the highest concentration was set at 50.0 w/v%. The lower concentrations were determined according to OECD TG 442B.
Preparation of vehicle: On the first application day, 10 mL of acetone and 2.5 mL of olive oil were mixed to prepare AOO.
Preparation of test substance formulation: On the first application day, 0.50009 g of the test substa nce was dissolved in AOO and filled up to 1 mL to make 50.0 w/v% solution. The lower concentration solutions were prepared by serial dilution method as follows. These solutions had been stored at the cold place (acceptable temperature: to 10°C) and used for 3 days.
Application: Twenty-five microliters of each solution was applied to the dorsum of each ear of the animals using a micropipette once a day for three consecution days.
Clinical observation: All the animals were observed more than once a day from the first application day (Day 1) to terminal day (Day 6). Erythema scoring was not performed since no erythema was observed.
Body weights: Body weights of all the animals were measured on Day 1 and Day 6 using an electric balance.
Ear thickness: Thicknesses of each ear were measured on Day 1 (before application), Day 3 and Day 6 using a digital caliper (Digimatic Caliper, Mitutoyo). Thickness of each ear was measured duplicate on each day, and the mean values were calculated.
Handling of animals after examinations: All the animals used in the pre-screen test were euthanized by cervical dislocation on Day 6.
Handling of dead or moribund animals: No moribund state or death occurred.
Evaluation of result: Dosages which induce following signs are supposed to be excluded from the main study.
-excessive irritation (erythema score 2: 3 and/or the ear thickness increases 25% or more)
-severe systemic toxicity and/or 5% or more body weight decrease

MAIN STUDY
Dose setting
In the pre-screen test, no abnormal changes which suggested systemic toxicity or excessive irritation were noted in any animals. Therefore, the dosages of the main study were set at 50.0, 25.0 and 10.0 w/v%.
Grouping
The test substance, vehicle control (AOO) and positive control (25.0 w/v% HCA) groups were set in the main study. Four animals were used in each group.
Preparations of vehicle, test substance formulation, positive control substance solution and BrdU so lution

Vehicle: On each sensitisation day, 10 mL of acetone and 2.5 mL of olive oil were mixed to prepare AOO.
Test substance formulation: On each sensitisation day, 0.500 g of test substance was dissolved in AOO and filled up to 1 mL to make 50.0 w/v% solution. The actual weights were 0.50033 g (Day 1), 0.50001 g (Day 2) and 0.50001 g (Day 3). The 25.0 and 10.0 w/v% solutions were prepared by the same method as pre-screen test.
Positive control substance solution: On the first sensitisation day, 0.25020 g of HCA was dissolved in AOO and filled up to 1 mL to make a 25.0 w/v% HCA solution under a yellow light condition. The solution was subdivided into three air-tight containers and the containers were shaded and preserved in refrigerator (acceptable temperature: 1-10 °C).
BrdU solution: Two days before the administration, 0.20002 g of 5-bromo-2'-deoxyuridine (BrdU, lot number M5H0079, Nacalai Tesque) was dissolved in physiological saline (lot number M6D75, O tsuka Pharmaceutical Factory) by ultrasonication and filled up to 20 mL to make 10 mg/mL solution. The BrdU solution was filtrated by a sterilizing filter (DISMIC®-25AS, pore size: 0.20 11m, lot num ber: 510221CD, Toyo roshi kaisha) and was stored in a sterile container in a refrigerator (acceptab le temperature: 1-10 °C) until administration. The preparation was conducted under a yellow light c ondition, and the sterilization and after procedures were conducted in a clean bench.

Sensitisation
The vehicle, test substance formulations and positive control solution were applied in the same way as the pre-screen test.
BrdU administration
Approximately 48 hours after the final sensitisation, 0.5 mL ofBrdU solution was administrated i ntraperitoneally to each animal using a syringe and a needle (Terumo).
Clinical observations
Clinical observations were performed in the same way as the pre-screen test except for the erythema scoring.
Body weights measurements
Body weights were measured in the same way as for pre-screen test. The mean and the standard deviation were calculated for each group on each measurement day.
Collection and weights measurement of auricular lymph nodes
Approximately 24 hours after the BrdU administration, the animals were euthanized by cervical dislocation and each auricular lymph node was taken. The lymph nodes were carefully dissected and trimmed of surrounding tissue and fat, and weighed both sides together. The mean value and the standard deviation of the lymph nodes weights were calculated for each group. The lymph nodes were stored individually in the biomedical freezer (acceptable temperature: -30 to -10 °C).
BrdU labelling index
The auricular lymph nodes were defrosted at room temperature, homogenized and suspended in physiological saline (lot number 6HOON, Otsuka Pharmaceutical Factory). This suspension was filtered by cell strainer (Becton, Dickinson and Company), and dispensed into 3 wells per animal of a 96 well microplate (Costar). The BrdU uptake quantity was measured by ELISA using a commercial kit (Cell Proliferation ELISA, BrdU colorimetric, lot number 17267000, Roche Diagnostics). Absorbance at 370 nm with a reference wavelength of 492 nm was measured using multidetection microplate reader (FLUOstar OPTIMA, BMG LABTECH). The mean value of the 3 wells was defined as BrdU labelling index.
Handling of dead or moribund animals
No death or moribund state occurred.
Evaluation of result
Calculation of stimulation index (SI)
The SI for individual animals of was calculated by dividing the BrdU labelling index for each animal by the mean value of BrdU labelling index for the vehicle control group. The SIs were rounded off to one decimal place, and mean SI of each group was calculated.
SI = BrdU labelling index for individual animal/Men value of BrdU labelling indices for 4 animals of the vehicle control group
Evaluation method
Test substance is regarded as a "sensitiser" when the SI of the test substance group is 2.0 or more,

and is regarded as a "non-sensitiser" when the SI of the test substance group is less than 1.6. When the SI is between 1.6-1.9, dose-response relationship and statistical significance are considered to evaluate the sensitisation potential of the test substance.

However in this study, the statistical analysis was not conducted since the Sis of the any test substance groups were less than 1.6.

Validity of study

The test is regarded as valid when the SI of the positive control group is 1.6 or more.

Positive control substance(s)

hexyl cinnamic aldehyde (CAS No 101-86-0)

Positive control results

The SI of the positive control group was 2.6.
The mean lymph node weights of the positive control group were 9.8 mg.
The mean BrdU labelling indices of the positive control group was 0.507.

Results

Key result
true
Parameter
SI
Value
>= 0.9 <= 1.0

Key result
true
Parameter
other: BrdU labelling indices
Value
> 0.168 < 0.196

Cellular proliferation data / Observations

The mean lymph nodes weights of the vehicle control group were 4.8 mg and those of 50.0, 25.0 and 10.0 w/v% test substance groups were 4.8, 4.7 and 5.8 mg, respectively.

Table 1 Clinical signs in the pre-screen test

Exp. Group			Animal No.	Observation period					
Group	Substance name	Concentration (w/v%)		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Test substance	PX-200	2.50	1	-	-	-	-	-	-
		5.00	2	-	-	-	-	-	-
		10.0	3	-	-	-	-	-	-
		25.0	4	-	-	-	-	-	-
		50.0	5	-	-	-	-	-	-

-: no abnormalities detected.

Table 2 Body weights in the pre-screen test

Exp. Group			Animal No.	Body weights (g)	
Group	Substance name	Concentration (w/v%)		Day 1	Day 6[a)]
Test substance	PX-200	2.50	1	21.0	21.2 (101)
		5.00	2	22.5	22.7 (101)
		10.0	3	22.7	23.3 (103)
		25.0	4	21.6	21.9 (101)
		50.0	5	21.0	21.2 (101)

a) Figures in parentheses indicate percentages compare to the initial body weight (Day 1).

Table 3 Thicknesses of auricle in the pre-screen test

Exp. Group			Animal No.	Thicknesses of auricle (mm)								
				Day 1			Day 3			Day 6		
Group	Substance name	Concentration (w/v%)		Left	Right	Average	Left	Right	Average[a)]	Left	Right	Average[a)]
Test substance	PX-200	2.50	1	0.18	0.19	0.19	0.17	0.18	0.18 (95)	0.16	0.18	0.17 (89)
		5.00	2	0.18	0.17	0.18	0.17	0.18	0.18 (100)	0.17	0.19	0.18 (100)
		10.0	3	0.15	0.18	0.17	0.16	0.17	0.17 (100)	0.17	0.18	0.18 (106)
		25.0	4	0.14	0.17	0.16	0.16	0.17	0.17 (106)	0.17	0.16	0.17 (106)
		50.0	5	0.16	0.17	0.17	0.16	0.17	0.17 (100)	0.18	0.17	0.18 (106)

a) Figures in parentheses indicate percentages compare to the initial thicknesses (Day 1).

Table 4 Clinical signs in the main study

Exp. Group			Animal No.	Observation period					
Group	Substance name	Concentration (w/v%)		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Vehicle control	AOO		1	-	-	-	-	-	-
			2	-	-	-	-	-	-
			3	-	-	-	-	-	-
			4	-	-	-	-	-	-
Positive control	HCA	25.0	5	-	-	-	-	-	-
			6	-	-	-	-	-	-
			7	-	-	-	-	-	-
			8	-	-	-	-	-	-
Test substance	PX-200	10.0	9	-	-	-	-	-	-
			10	-	-	-	-	-	-
			11	-	-	-	-	-	-
			12	-	-	-	-	-	-
		25.0	13	-	-	-	-	-	-
			14	-	-	-	-	-	-
			15	-	-	-	-	-	-
			16	-	-	-	-	-	-
		50.0	17	-	-	-	-	-	-
			18	-	-	-	-	-	-
			19	-	-	-	-	-	-
			20	-	-	-	-	-	-

AOO: acetone:olive oil (4:1 v/v)
HCA: α-hexylcinnamaldehyde
-: no abnormalities detected.

Table 6 Lymph node weights in the main study

Exp. Group			Animal No.	Lymph node weights (mg)	
Group	Substance name	Concentration (w/v%)		Individual	Mean ± S.D.
Vehicle control	AOO		1	4.8	4.8 ± 0.4
			2	5.1	
			3	5.0	
			4	4.2	
Positive control	HCA	25.0	5	9.1	9.8 ± 0.7
			6	9.3	
			7	10.3	
			8	10.5	
Test substance	PX-200	10.0	9	6.3	5.8 ± 0.6
			10	5.7	
			11	5.0	
			12	6.2	
		25.0	13	3.5	4.7 ± 1.3
			14	4.7	
			15	4.2	
			16	6.5	
		50.0	17	4.8	4.8 ± 0.3
			18	5.1	
			19	4.9	
			20	4.5	

S.D.: standard deviation
AOO: acetone:olive oil (4:1 v/v)
HCA: α-hexylcinnamaldehyde

Table 7 BrdU labelling indices and stimulation indices in the main study

Exp. Group			Animal No.	BrdU labelling indices		Stimulation indices	
Group	Substance name	Concentration (w/v%)		Individual	Mean ± S.E.	Individual	Mean ± S.E.
Vehicle control	AOO		1	0.152	0.194 ± 0.024	0.8	1.0 ± 0.1
			2	0.247		1.3	
			3	0.155		0.8	
			4	0.222		1.1	
Positive control	HCA	25.0	5	0.613	0.507 ± 0.052	3.2	2.6 ± 0.3
			6	0.425		2.2	
			7	0.577		3.0	
			8	0.411		2.1	
Test substance	PX-200	10.0	9	0.174	0.196 ± 0.018	0.9	1.0 ± 0.1
			10	0.234		1.2	
			11	0.216		1.1	
			12	0.159		0.8	
		25.0	13	0.187	0.192 ± 0.019	1.0	1.0 ± 0.1
			14	0.189		1.0	
			15	0.151		0.8	
			16	0.241		1.2	
		50.0	17	0.156	0.168 ± 0.004	0.8	0.9 ± 0.1
			18	0.168		0.9	
			19	0.169		0.9	
			20	0.177		0.9	

S.E.: standard error
AOO: acetone:olive oil (4:1 v/v)
HCA: α-hexylcinnamaldehyde

Interpretation of results
GHS criteria not met

Conclusions
In the main study, no abnormal findings which suggested excessive irritation or severe systemic toxicity were observed in clinical signs or body weight changes in any treatment groups. Therefore, all the data obtained were used for the assessment of skin sensitisation potential. As a result, the Sis of the 50.0, 25.0 and 10.0 w/v% were 0.9, 1.0 and 1.0, respectively: the Sis of any dose levels were less than 1.6.

Therefore, under the conditions tested, PX-200 was considered to be a non-sensitiser. As for the positive control group, the SI was 2.6 and thus the study was confirmed to be valid.

3.7.1.3 [Study 3]

Study reference:
Anonymous (2008)

Detailed study summary and results:
Test type
Skin sensitisation: in vivo (non-LLNA)
the study design followed the principles of the Buehler sensitisation method with three inductions of 10 guinea pigs one week apart followed by a challenge of the 28 days after the first induction and skin reaction recorded to determine the immunological dermal response.

Test substance
- Tetrakis(2,6-dimethylphenyl)-m-phenylene biphosphate / 139189-30-3 / 432-770-2.

Species
guinea pig

Strain
Hartley guinea pig
Sex
Female

Induction

Route epicutaneous, occlusive **Vehicle** propylene glycol
Concentration / amount Test sample for the induction phaseThe test sample was suspended in PG to prepare 50 w/v % test solution. Test sample for the challenge phase The test sample was suspended in PG to prepare 25 w/v % test solution.

Challenge

Route epicutaneous, occlusive **Vehicle** propylene glycol
Concentration / amount Test sample for the induction phaseThe test sample was suspended in PG to prepare 50 w/v % test solution. Test sample for the challenge phase The test sample was suspended in PG to prepare 25 w/v % test solution.

No. of animals per dose
10 test 5 control animals

Details on study design
RANGE FINDING TESTS:MAIN STUDY
A. INDUCTION EXPOSURE
- No. of exposures: 3
- Exposure period: 28 days
- Test groups: 10 animals
- Control group: 5 animals
- Site: left flank
- Frequency of applications: Days 1, 8 and 15
- Duration: continued for 6 hours
- Concentrations: 50 w/v %

B. CHALLENGE EXPOSURE
- No. of exposures: 1
- Day(s) of challenge: 29
- Exposure period: continued for 6 hours
- Test groups: 10 animals
- Control group: 5 animals
- Site: right flank
- Concentrations: 25 w/v %
- Evaluation (hr after challenge): 24 and 48 hours

Challenge controls
Induction with Propylene Glycol only on the left flank and challenge of 25 w/v % test solution on the right flank

Positive control substance(s)
no

Administration/exposure

- Control group and treatment
  - Not specified
- Route of induction and challenge induction
  - Not applicable
- Induction
  - Not applicable
- Challenge
  - Not applicable

Results and discussion

Key result
true
Reading
1st reading
Hours after challenge
24.0
Group
test group
Dose level
25 %w/v
No. with + reactions
0

Total no. in group
10
Remarks on result
other: Reading: 1st reading. . Hours after challenge: 24.0. Group: test group. Dose level: 25 %w/v. No with. + reactions: 0.0. Total no. in groups: 10.0.

Key result
true
Reading
2nd reading
Hours after challenge
48.0
Group
test group
Dose level
25%w/w
No. with + reactions
0
Total no. in group
10
Remarks on result
other: Reading: 2nd reading. . Hours after challenge: 48.0. Group: test group. Dose level: 25%w/w.

No with. + reactions: 0.0. Total no. in groups: 10.0.

Non-sensitiser
Stimulation index: >=0.9 - <=1
BrdU labelling indices: >0.168 - <0.196

Interpretation of results
not sensitising
Criteria used for interpretation of results: EU

Conclusions
The substance is considered to be not sensitising under the conditions of the study

3.7.2 Human data

3.7.2.1 [Study 1]

Study reference:

Pessoto Rosa, V. 2017

Test guideline

Qualifier
equivalent or similar to
Guideline
other: KLIGMAN, A.M. & WOODING, W.M.
Version / remarks
KLIGMAN, A.M. & WOODING, W.M. A method for the measurement and evaluation of irritants of h uman skin. J. Invest.. Derm. 49: 78-94, 1967.
Deviations
not specified

Method

Type of population
general
Ethical approval
confirmed and informed consent free of coercion received
Subjects
Population Description
A total of 92 study subjects were recruited for this study. Out of those, 22 subjects (003, 004, 006, 008, 009, 011, 016, 021, 030, 031, 033, 037, 041, 048, 051, 054, 056, 059, 061, 076, 078 and 083) did not meet the inclusion criteria or presented any of the exclusion criteria.
The study was initiated with 70 subjects, being 63 female and 07 male subjects, aged from 18 to 67 Years

Clinical history

Inclusion Criteria
-Healthy study subjects;
-Intact skin on test site;
-Agreement to adhere to the procedures and requirements of the study and to report to the institute on the day(s) and at the time(s) scheduled for the assessments;
-Ability of giving a written consent for participating in the study;
-Aged from 18 to 70 years old;
-Study subjects of any gender;
-Phototype (Fitzpatrick): I to IV.

Non Inclusion Criteria
-Any skin marks on the test site that might interfere with the assessment of possible skin reactions (pigmentation disorders, vascular malformations, scars, increased pilosity, and great amounts of ephelides and nevus, sunburns);
-Active dermatosis (local or disseminated) that might interfere with the results of the study;
-Pregnancy or breastfeeding;
-Previous history of allergic reactions, irritation or intense feelings of discomfort to topical -use products, cosmetics or medication;
-Subjects with history of allergy to the material used in the study;
-Previous history of atopy;
-History of pathologies aggravated or triggered by ultraviolet radiation;
-Subjects suffering from immunodeficiencies;
-Intense exposure to sunlight or to sun tanning sessions up to 15 days before the initial evaluation;
-Intention of being intensely exposed to sunlight or to sun tanning sessions during the study period;
-Intention of sea bathing, going to the pool or sauna during the study;
-Subjects who practice water sports;
-Dermographism;
-Use of the following topical or systemic medications: immunosuppressive drugs, antihistamines, nonhormonal anti-inflammatory drugs, and corticosteroids within two weeks before the selection process;
-Oral or topical treatment with vitamin A acid and/or its derivatives up to 1 month before the study start;
-Aesthetic and/or dermatological treatment performed on the body within 03 weeks before selection;
-Intention of being vaccinated during the study period or up to 3 weeks before the study;
-Any conditions which the investigator finds compromising to the evaluation of the study;
-History of lack of adherence or unwillingness to adhere to the study protocol;
-Professionals who are directly involved in the performance of the current protocol as well as their relatives.

Route of administration
dermal

Details on study design
Study Design
Clinical, controlled and single-blind study.

Materials and Equipment
-Adhesive hypoallergenic card for patch testing with duly identified 1.0-cm^2 filter paper discs;
-Semi-occlusive hypoallergenic tape;
-0.9% sterile physiological solution (NaCl 0.9%);
-Gloves, masks and caps;
-Surgical marker;
-Cotton swab;
-Scale
-Micropipette;
-Heating plate;
-Beaker;
-Dropper bottle;

-Transparent bottle.

Test Site
The product was applied to the study subjects back (scapular area).
Population Size
This study was conducted with 70 approved subjects so it could be completed with at least 50 responses.
Procedures
At first the study subjects were assessed by a dermatologist in order to verify the inclusion and exclusion criteria.

The patch test methodology (Kligman & Wooding, 1967), also known as contact test or epi cutaneous test, was used.

The product (0.05g/cm²) was distributed on the duly identified patch test filter paper disc and the 0.9% sterile physiological solution, used as control, was distributed on another duly identified disc.
The patch test containing both the test-product and control was then attached to the scapular area of the right or left back of the subjects.

Induction Period: the applications were performed three times a week, for three consecutive weeks, remaining in contact with the skin for 48 hours during the week and for 72 hours during weekends.
Rest Period: There was a rest period of at least 10 days following the induction period, when no patches were applied.
Challenge period: After the rest period, a patch with the test product and control was applied to the right or left back of the subjects on a virgin area, that is, where no patches had been applied before.
The patch was removed by the investigators after approximately 48 hours of contact with the skin.
The assessments (readings) were performed approximately 30 minutes (48h reading) and 24 hours (72h reading) after patch test removal.
The subjects were assessed at the end of the study by a dermatologist and supervised all along the study.
Assessment of Clinical Signs (Readings)

In case any subject presented any clinical sign during the readings, the assessment scale published by the International Contact Dermatitis Research Group - ICDRG (FISHER, 1995) would be used.

Scale published by the International Contact Dermatitis Research Group – ICDRG

REACTION	RESULT
0 – Absent	Negative (-)
1 - Mild Erythema	Doubtful (?)
2 - Clear Erythema	Positive (+)
3 - Erythema + Edema + Papules	Positive (++)
4 - Erythema + Edema + Papules + Vesicles	Positive (+++)

Study Schedule:

			Stages				
			Sign Informed Consent	Clinical Assessment by the Dermatologist	Patch test Application	Patch test Removal	Assessments (Readings)
		Visit 1	X	X	-	-	-
Induction Period	Week 1	Visit 2	-	-	X	-	-
		Visit 3	-	-	X	X	X
		Visit 4	-	-	X	X	X
	Week 2	Visit 5	-	-	X	X	X
		Visit 6	-	-	X	X	X
		Visit 7	-	-	X	X	X
	Week 3	Visit 8	-	-	X	X	X
		Visit 9	-	-	X	X	X
		Visit 10	-	-	X	X	X
		Visit 11	-	-	-	X	X
Rest period - weeks 4 and 5 – no visits performed							
Challenge Period	Week 6	Visit 12	-	-	X	-	-
		Visit 13	-	-	-	X	X
		Visit 14	-	X	-	-	X

Results of examinations

Adherence to the Study

A total of 58 subjects completed the study.

A total of 09 subjects withdrew from the study due to personal reasons unrelated to the test product (025, 026, 045, 057, 079, 086, 087, 089 and 090).

Subjects 007, 013 and 014 presented irritation resulting from the exposure of the semi-occlusive tape (sticking plaster), probably due to individual pre-disposition, and for that reason the applications were discontinued and their data were not used in the study.

Dermatological Clinical Assessment

During the study, no subjects presented any clinical signs in the test product application site.

No subjects presented clinical signs in the control site.

Patch test assessments: Test-product

Subject No	Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading	Application	Reading	Reading
001	0	0	0	0	0	0	0	0	0	0	0	0	0
002	0	0	0	0	0	0	0	0	0	0	0	0	0
005	0	0	0	0	0	0	0	0	0	0	0	0	0
007	0	0	0	0	0	0	0	R	R	R	R	R	R
010	0	0	0	0	0	0	0	0	0	0	0	0	0
012	0	0	0	0	0	0	0	0	0	0	0	0	0
013	0	0	0	0	0	0	R	R	R	R	R	R	R
014	0	0	0	0	0	0	R	R	R	R	R	R	R
015	0	0	0	0	0	0	0	0	0	0	0	0	0
017	0	0	0	0	0	0	0	0	0	0	0	0	0
018	0	0	0	0	0	0	0	0	0	0	0	0	0
019	0	0	0	0	0	0	0	0	0	0	0	0	0
020	0	0	0	0	0	0	0	F	0	0	0	0	0
022	0	0	0	0	0	0	0	0	0	0	0	0	0
023	0	0	0	0	0	0	0	0	0	0	0	0	0
024	0	0	0	0	0	0	0	0	0	0	0	0	0
025	F/R	R	R	R	R	R	R	R	R	R	R	R	R
026	0	0	0	F	0	0	F/R	R	R	R	R	R	R
027	0	0	0	0	0	0	0	0	F	0	0	0	0
028	0	0	0	0	0	0	0	0	0	0	0	0	0
029	0	0	0	0	0	F	0	0	0	0	0	0	0
032	0	0	0	0	0	0	0	0	0	F	0	0	0
034	0	0	0	0	0	0	0	0	0	0	0	0	0
035	0	0	0	0	0	0	0	0	0	0	0	0	0
036	0	0	0	0	0	0	0	0	0	0	0	0	0
038	0	0	0	0	0	0	0	0	0	0	0	0	0
039	0	0	0	0	0	0	0	0	0	0	0	0	0
040	0	0	0	0	0	0	0	0	0	0	0	0	0
042	0	0	0	0	F	0	0	0	0	0	0	0	0
043	0	0	0	0	0	0	0	0	0	0	0	0	0
044	0	0	0	0	0	0	0	0	F	0	0	0	0
045	F/R	R	R	R	R	R	R	R	R	R	R	R	R
046	0	0	0	0	0	0	0	0	0	0	0	0	0
047	0	0	0	0	0	0	0	0	0	0	0	0	0
049	0	0	0	0	0	0	0	0	0	0	0	0	0

Caption:
X = Not Applied / Reading Not Performed
F = Absence
R = Removed from the Study
DK = Darkening
DY = Dryness
F/R = Absence / Removed from the Study

0= No reaction
1 = Mild Erythema
2 = Clear Erythema
3 = Erythema + Edema + Papules
4 = Erythema + Edema + Papules + Vesicles

Subject No	Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading + Application	Reading	Application	Reading	Reading
050	0	0	0	0	0	0	0	0	0	0	0	0	0
052	0	0	0	0	0	0	0	0	0	0	0	0	0
053	0	0	0	0	0	0	0	0	0	0	0	0	0
055	0	0	0	0	0	0	0	0	0	0	0	0	0
057	0	0	F/R	R	R	R	R	R	R	R	R	R	R
058	0	0	0	0	0	0	0	0	0	0	0	0	0
060	0	0	0	F	0	0	0	0	0	0	0	0	0
062	0	0	0	0	0	0	0	0	0	0	0	0	0
063	0	0	0	0	0	0	0	0	F	0	0	0	0
064	0	0	0	0	0	0	F	0	0	0	0	0	0
065	0	0	0	0	0	0	0	F	0	0	0	0	0
066	0	0	0	0	0	0	0	0	0	0	0	0	0
067	0	0	0	0	0	0	0	0	0	0	0	0	0
068	0	0	0	0	0	0	0	F	0	0	0	0	0
069	0	0	0	0	0	0	0	0	F	0	0	0	0
070	0	0	0	0	0	0	0	0	0	0	0	0	0
071	0	0	0	0	0	0	0	0	0	0	0	0	0
072	0	0	0	0	0	0	0	0	0	0	0	0	0
073	0	0	0	0	0	F	0	0	0	0	0	0	0
074	0	0	0	0	0	0	0	0	0	0	0	0	0
075	0	0	0	0	0	0	0	0	0	0	0	0	0
077	0	0	0	0	0	0	0	0	0	0	0	0	0
079	0	0	0	F	F/R	R	R	R	R	R	R	R	R
080	0	0	0	0	0	0	0	0	0	0	0	0	0
081	0	0	0	0	0	0	0	0	0	0	0	0	0
082	0	0	0	0	0	0	0	0	0	0	0	0	0
084	0	0	0	0	0	0	0	0	0	0	0	0	0
085	0	0	0	0	0	0	0	0	0	0	0	0	0
086	0	0	0	F	0	0	0	0	F/R	R	R	R	R
087	F/R	R	R	R	R	R	R	R	R	R	R	R	R
088	0	0	0	0	0	0	0	0	0	0	0	0	0
089	F/R	R	R	R	R	R	R	R	R	R	R	R	R
090	F/R	R	R	R	R	R	R	R	R	R	R	R	R
091	0	0	0	0	0	0	0	0	F	0	0	0	0
092	0	0	0	0	0	0	0	0	0	0	0	0	0

Caption:
X = Not Applied / Reading Not Performed
F = Absence
R = Removed from the Study
DK = Darkening
DY = Dryness
F/R = Absence / Removed from the Study

0= No reaction
1 = Mild Erythema
2 = Clear Erythema
3 = Erythema + Edema + Papules
4 = Erythema + Edema + Papules + Vesicles

Conclusions

According to the methodology used to assess the skin sensitisation potential of the product PX-200, submitted by the company DAIHACHI CHEMICAL INDUSTRY CO.,LTD. it could be concluded that:
-During the study, no subjects presented skin clinical signs related to the product.
-The product did not induce a skin sensitisation process in the study group.
-The product was considered safe under the study conditions.

3.7.3 Other data

3.7.3.1 [Study 1]

Study reference:

Chevallier, K. 2017a

Qualifier
according to

Guideline
OECD Guideline 442C (In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA))

Version / remarks
OECD guideline No. 442C: in chemico skin sentitization: Direct Peptide Reactivity Assay (DPRA), adopted on 04 February 2015.

Deviations
yes See "Any other information" for details

GLP compliance
yes (incl. certificate)

Type of study
other: Direct Peptide Reactivity Assay (DPRA)

Test substance

- PX-200, Form: solid: particulate/powder

Details on study design
DESIGN OF THE DIRECT PEPTIDE REACTIVITY ASSAY
The test item was tested in one run. The run was processed as described below.
Preparation of the samples
The following samples were prepared in triplicate except for the co-elution control samples for which only one sample was prepared per peptide buffer.

Co-elution control samples preparation

For the co-elution control with cysteine peptide: 50 µL of test item formulation was incubated with 750 µL of cysteine peptide dilution buffer (without cysteine peptide) and 200 µL of acetonitrile.
For the co-elution control with lysine peptide: In parallel, 250 µL of test item formulation was incubated with 750 µL of lysine peptide dilution buffer (without lysine peptide).

Reference control samples preparation
Reference control A and B samples: In a vial, acetonitrile was added to a volume of peptide solution (cysteine or lysine) to achieve a nominal concentration of 0.500 mM.
Reference control C samples: Reference control C samples were prepared for each solvent used to dissolve the test and positive control items.
For the reference control C prepared with cysteine peptide: 50 µL of vehicle (acetonitrile) was incubated with 750 µL of cysteine peptide solution (at 0.667 mM in phosphate buffer at pH 7.5) and 200 µL of acetonitrile.
For the reference control C prepared with lysine peptide: In parallel, 250 µL of vehicle (acetonitrile) was incubated with 750 µL of lysine peptide solution (at 0.667 mM in ammonium acetate buffer at pH 10.2).

Cinnamaldehyde (positive control) depletion control samples preparation
For the reactivity of cinnamaldehyde with cysteine peptide: 50 µL of cinnamaldehyde at 100 mM in acetonitrile was incubated with 750 µL of cysteine peptide solution (at 0.667 mM in phosphate buffer at pH 7.5) and 200 µL of acetonitrile.
For the reactivity of cinnamaldehyde with lysine peptide: In parallel, 250 µL of cinnamaldehyde at 100 mM in acetonitrile was incubated with 750 µL of lysine peptide solution (at 0.667 mM in ammonium acetate at pH 10.2).

Test item samples preparation
For the reactivity of test item with cysteine peptide: 50 µL of test item formulation was incubated with 750 µL of cysteine peptide solution (at 0.667 mM in phosphate buffer at pH 7.5) and 200 µL of acetonitrile.

For the reactivity of test item with lysine peptide: In parallel, 250 µL of test item formulation was incubated with 750 µL of lysine peptide solution (at 0.667 mM in ammonium acetate at pH 10.2).

Incubation of the samples
All samples (co-elution controls, reference controls, test item and positive control samples) were then incubated during 24 (± 2) hours at 25 °C and protected from light before injection into the HPLC/UV system.
At the end of the incubation period, a visual inspection of the samples was performed prior to HPLC analysis to detect precipitate or phase separation.
Samples presenting precipitate were centrifuged at 400g for a period of 5 minutes at room temp erature or at +4 °C and only supernatants were then injected onto the HPLC/UV system. Otherwise, the vials were directly transferred onto the HPLC/UV system.

Preparation of the calibration curve samples
One set of calibration standards was prepared with each analytical sequence by spiking each peptide (lysine and cysteine) in separate solutions of 20% acetonitrile:peptide dilution buffer to obtain at least six different concentration levels ranging from 0.0167 to 0.534 mM. A dilution buffer blank was also included in the standard calibration curve.

The calibration curves were defined by the relationships between the peak area signal of the peptide versus the nominal concentration. These curves were obtained by using the appropriate mathematical model.

HPLC/UV analysis of the samples
The study samples were assayed in batches using HPLC/UV analysis.
For each peptide, the analytical sequence included at least:
-one blank sample (peptide dilution buffer),
-one calibration curve injected at the beginning of the analytical batch,
-three reference control A samples,
-the co-elution control sample,
-three reference control B samples,
-reference control C samples (replicate 1),

-positive control sample (replicate 1),
-test item study sample (replicate 1).
The injection order of the reference control C, positive control and test item study samples were reproduced identically for replicate 2 and then replicate 3:
-three reference control B samples.

STUDY PLAN ADHERENCE
The study was performed in accordance with study plan No. 44582 TIR, with the following deviation from the agreed study plan:
. § Incubation of the samples: after the incubation dated 15 December 2016, samples presenting precipitate were centrifuged at 400g for a period of 5 minutes in a centrifuge programmed at +4 °C instead of at room temperature (by operating error). Thus the temperature of the tank was at room temperature in the beginning and at +6 °C at the end of the centrifugation. The duration was considered to be too short to have an impact on the samples. As a consequence, this deviation was considered not to have compromised the validity or integrity of the study.

Results and discussion

Key result
true

Parameter
other: peptide reactivity

Value

0.36

Vehicle controls valid
yes

Negative controls valid
not applicable

Positive controls valid
yes

Remarks on result
no indication of skin sensitisation

Percent peptide depletion for the test item samples

DETERMINATION OF CYSTEINE PEPTIDE AND LYSINE PEPTIDE DEPLETION IN SAMPLES SPIKED WITH A SOLUTION AT 100 mM OF PX-200

Sample number	Cysteine peptide		Lysine peptide		Mean depletion rate (%) of PX-200	Depletion classification
	Peak area (µV/sec)	% depletion	Peak area (µV/sec)	% depletion		
1	2596654	0.61	2085878	0.00*		
2	2593066	0.75	2088116	0.00*		
3	2601841	0.41	2053145	0.38		
Mean	-	**0.59**	-	**0.13**	0.36	No reactivity/ minimal reactivity
SD	-	**0.17**	-	**0.22**		
% CV	-	**28.5**	-	**173.2**		
Precipitate:	**Yes**		**Yes**			
Micelle	**No**		**No**			

*: Value set to 0 due to negative depletion

-: not applicable

DETERMINATION OF CYSTEINE PEPTIDE AND LYSINE PEPTIDE CONCENTRATION IN REFERENCE CONTROL C SAMPLES PREPARED IN ACETONITRILE

	Cysteine peptide			Lysine peptide		
Sample number	Peak Area (µV/sec)	Concentration (mM)	%Dev	Peak Area (µV/sec)	Concentration (mM)	%Dev
1	2619032	0.499	*(-0.1)*	2065090	0.498	*(-0.4)*
2	2613119	0.498	*(-0.3)*	2060532	0.497	*(-0.6)*
3	2605814	0.497	*(-0.6)*	2057403	0.496	*(-0.7)*
Mean	**2612655**	**0.498**	***(-0.4)***	**2061008**	**0.497**	***(-0.6)***
SD	-	**0.001**	-	-	**0.001**	-
% CV	-	**0.3**	-	-	**0.2**	-

DETERMINATION OF % INTERFERENCE DUE TO CO-ELUTION OF PX-200 WITH CYSTEINE OR LYSINE PEPTIDES

	Peak detected at the cysteine retention time		Peak detected at the lysine retention time	
Sample number	Peak Area (µV/sec)	% Interference	Peak Area (µV/sec)	% Interference
1	0	*(0.0)*	0	*(0.0)*
Precipitate:	**Yes**		**Yes**	
Micelle	**No**		**Yes**	

Percent peptide depletion for the positive control samples

DETERMINATION OF CYSTEINE PEPTIDE AND LYSINE PEPTIDE DEPLETION IN SAMPLES SPIKED WITH A SOLUTION AT 100 mM OF CINNAMALDEHYDE

Sample number	Cysteine peptide		Lysine peptide		Mean depletion rate (%) of Cinnamaldehyde	Depletion classification
	Peak area (µV/sec)	% depletion	Peak area (µV/sec)	% depletion		
1	728662	72.11	913821	55.66		
2	751271	71.24	922619	55.23		
3	743516	71.54	962281	53.31		
Mean	-	**71.63**	-	**54.74**		
SD	-	**0.44**	-	**1.25**	**63.18**	High reactivity
% CV	-	**0.6**	-	**2.3**		

DETERMINATION OF CYSTEINE PEPTIDE AND LYSINE PEPTIDE CONCENTRATION IN REFERENCE CONTROL C SAMPLES PREPARED IN ACETONITRILE

Sample number	Cysteine peptide			Lysine peptide		
	Peak Area (µV/sec)	Concentration (mM)	%Dev	Peak Area (µV/sec)	Concentration (mM)	%Dev
1	2619032	0.499	*(-0.1)*	2065090	0.498	*(-0.4)*
2	2613119	0.498	*(-0.3)*	2060532	0.497	*(-0.6)*
3	2605814	0.497	*(-0.6)*	2057403	0.496	*(-0.7)*
Mean	**2612655**	**0.498**	***(-0.4)***	**2061008**	**0.497**	***(-0.6)***
SD	**-**	**0.001**	**-**	**-**	**0.001**	**-**
% CV	**-**	**0.3**	**-**	**-**	**0.2**	**-**

-: not applicable

The acceptance criteria for the calibration curve samples, the reference and positive controls as well as for the study samples were satisfied. The study was therefore considered to be valid.

Analysis of the chromatograms of the co-elution samples (Figures 1 and 4) indicated that the test item did not co-elute with either the lysine or the cysteine peptides. As a result, the mean percent depletion values were calculated for each peptide:

-for the cysteine peptide, the mean depletion value was 0.59%,
-for the lysine peptide, the mean depletion value was 0.13%.

The mean of the percent cysteine and percent lysine depletions was equal to 0.36%. Accordingly, the test item was considered to have no/minimal peptide reactivity. Therefore, the DPRA prediction is considered as negative and the test item may have no potential to cause skin sensitisation.

Since precipitate and/or phase separation (micelles) were observed at the end of the incubation with the peptides, the peptide depletion may be underestimated. Therefore, the conclusion on the lack of reactivity cannot be drawn with sufficient confidence.

Interpretation of results
GHS criteria not met

Conclusions

Under the experimental conditions of the study, the test item PX-200, was considered to have no/minimal peptide reactivity, though with limitations due to test item precipitation or phase separation. The test item is considered negative in the DPRA assay.

3.7.3.2 [Study 2]

Study reference:
Chevallier, K 2017b

Detailed study summary and results:
Test type
in vitro study

Qualifier
according to

Guideline
OECD Guideline 442D (In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method)

Version / remarks
OECD Guideline 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, adopted on February 2015.

Deviations
no

GLP compliance
yes (incl. certificate)

Type of study
other: ARE-Nrf2 Luciferase Test

Test substance
- PX-200, Form: solid: particulate/powder

Details on study design
STUDY DESIGN
The test item was tested in two independent runs using cells from a different passage number. The plates were processed as described below.

Solubility assay
A solubility assay was performed prior the first treatment in order to select the vehicle (among DMSO, water or treatment culture medium). Sonication for 5 minutes and heating up to 80 °C for 40 minutes were used in order to improve the solubility of the test item.

Since the test item was found soluble in DMSO at 200 mM after 5 minutes of sonication and 40 minutes of heating at 80 °C, this stock formulation was diluted in treatment culture medium to the final concentration of 2000 µM. Then, a visual inspection of the sample was performed to evaluate the presence of precipitate.

Method for a run of KeratinoSens™ assay
Cell seeding for testing
-Cells were grown using general culture procedures up to 80-90% confluence,

-the day prior to treatment, cells were washed twice with D-PBS containing 0.05% EDTA, harvested, re-suspended in Maintenance medium No. 2 and counted using Trypan Blue dye. Cell concentration was adjusted to a density of 8 x 104 cells/mL,
-cells were then distributed into four 96-well plates (three white plates and one transparent plate), by adding 125 µL (representing 1 x 104 cells) per well taking care to avoid sedimentation of the cells during seeding,
-after seeding, the cells were grown for 24 (± 1) hours in the 96-well microtiter plates prior to test item addition.

Treatment
-After the 24-hour growing period, the medium was removed by aspiration and replaced by 150 µL of treatment medium,
-from the Master plate 4x, a volume of 50 µL was added to each well of the three white assay plates and 50 µL to the transparent plate for the cytotoxicity evaluation,
-all plates were covered by a sealing membrane to avoid evaporation of volatile test items and to avoid cross-contamination between wells,
-the plates were then incubated for 48 (± 2) hours at 37 °C, 5% CO2, 90% humidity.

Endpoint measurements
Microscopic observation to evaluate the presence or absence of precipitate - transparent plate
-After the 48 (± 2) hours incubation period, the presence or absence of precipitate/emulsion was determined in each well by microscopic inspection.
Luminescence flash signal to evaluate induction signal - white plates
-After incubation, the supernatants from the white assay plates were discarded,
-the cells were washed once with D-PBS,
-a volume of 20 µL of passive lysis buffer was added to each well and the cells were incubated for 20 (± 2) minutes at room temperature and under orbital shaking,
-the plates containing the passive lysis buffer were then placed in the luminometer for reading using the following program:
- 50 µL of the luciferase substrate was added to each well,
- 1 second after this addition, the luciferase signal was integrated for 2 seconds.
Absorbance signal to evaluate the cytotoxicity - transparent plate
-For the cell viability assay plate, the medium was replaced by 200 µL of treatment medium,
-a volume of 27 µL of a MTT solution at 5 mg/mL in D-PBS was then added to each well of the t ransparent 96-well plate,
-the plates were covered with a sealing membrane and returned at 37 °C in the incubator in humidified atmosphere for 4 hours (± 10 minutes),
-at the end of the incubation period, the medium was removed and a volume of 200 µL of a 10% SDS solution was added to each well,
-the plates were covered with a sealing membrane and placed at 37 °C in the incubator in humidified atmosphere for an overnight period to extract the formazan from cells,
-after the overnight incubation, the absorption of each well was determined at 600 nm using the plate reader.

Results and discussion

Positive control results
Second run
The criterion "the average induction (Imax) in the three replicate plates for the positive control at 64 µM should be between 2 and 8" was not fulfilled (i.e. Imax of 10.66). However, since a clear dose response with increasing luciferase activity at increasing concentrations was obtained for the positive control, this was considered not to have any impact on the validity of the results of this run.

Results

Key result
true

Parameter
other: cell viability

Value

> 70.0

Vehicle controls valid
yes

Negative controls valid
not applicable

Positive controls valid
yes

Remarks on result
no indication of skin sensitisation

Other effects / acceptance of results
No geometric mean IC30 or IC50 was calculated since the cell viability was > 70% in both runs.
The evaluation criteria for a negative response are met in both runs, the final outcome is therefore negative.
Since precipitate was observed in the test item-treated wells at the end of the 48-hour treatment period, the luciferase activity may be underestimated. Therefore, the conclusion on the lack of activity cannot be drawn with sufficient confidence.

This negative result can be used to support the discrimination between skin sensitisers and non-sensitisers in the context of an integrated approach to testing and assessment. It cannot be used on its own to conclude on a skin sensitisation potential.

Evaluation of the viability (%) of cultures treated with the test item for each run

Concentrations (µM)

PX-200	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500	1000	2000
Viability (%) in Run 1	116	134	140	133	135	136	126	127	120	121	140	124
Viability (%) in Run 2	105	110	108	108	106	108	111	95	94	91	111	113
Mean viability (%)	**110**	**122**	**124**	**121**	**120**	**122**	**118**	**111**	**107**	**106**	**125**	**118**
Geometric Mean (%)	**110**	**122**	**123**	**120**	**120**	**121**	**118**	**110**	**107**	**105**	**125**	**118**
SD	**7**	**17**	**23**	**18**	**20**	**19**	**10**	**23**	**18**	**21**	**20**	**8**

Gene induction values, I_{max}, IC_{30}, IC_{50} and $EC_{1.5}$ values, mean and SD values obtained after treatment with the test item in each run

Concentrations (µM)

PX-200	*0.98*	*1.95*	*3.91*	*7.81*	*15.63*	*31.25*	*62.5*	*125*	*250*	*500*	*1000*	*2000*
Induction values in Run 1	0.9	0.9	1.0	0.9	0.9	1.0	0.9	0.8	0.7	0.7	0.8	0.7
Induction values in Run 2	0.7	0.9	0.8	0.9	0.8	0.8	0.8	0.8	0.7	0.8	0.8	0.7
Mean induction	**0.8**	**0.9**	**0.9**	**0.9**	**0.8**	**0.9**	**0.9**	**0.8**	**0.7**	**0.8**	**0.8**	**0.7**
SD	**0.1**	**0.0**	**0.1**	**0.0**	**0.0**	**0.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

Imax and $EC_{1.5}$ results

PX-200	Imax	$EC_{1.5}$ (µM)	IC_{50} (µM)	IC_{30} (µM)
Run 1	0.99	-	-	-
Run 2	0.88	-	-	-
Mean	**0.94**	**n.r.**	**n.r.**	**n.r.**
Geometric Mean	**n.r.**	**-**	**-**	**-**
SD	**0.08**	**-**	**-**	**-**

- : no data available

n.r.: not requested by the OECD Guideline

Evaluation of the viability (%) of cultures treated with the positive control for each run

Concentrations (µM)

cinnamic aldehyde	*4*	*8*	*16*	*32*	*64*
Viability (%) in Run 1	112	127	116	132	144
Viability (%) in Run 2	93	112	118	126	75
Mean viability (%)	**103**	**119**	**117**	**129**	**110**
Geometric Mean (%)	**102**	**119**	**117**	**129**	**104**
SD	**13**	**11**	**2**	**4**	**49**

Gene induction values, I_{max}, IC_{30}, IC_{50} and $EC_{1.5}$ values obtained with the positive control for each run

	Concentrations (µM)								
cinnamic aldehyde	*4*	*8*	*16*	*32*	*64*	**Imax**	**$EC_{1.5}$ (µM)**	**IC_{50} (µM)**	**IC_{30} (µM)**
Run 1	1.4	1.3	1.7	2.6	5.6	5.56	11.68	-	-
Run 2	1.1	1.4	1.9	2.8	10.7	10.66	9.50	-	-
Mean	**1.3**	**1.4**	**1.8**	**2.7**	**8.1**	**8.11**	**n.r.**	**n.r.**	**n.r.**
Geometric Mean	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**n.r.**	**10.53**	**-**	**-**
SD	**0.2**	**0.1**	**0.1**	**0.2**	**3.6**	**3.60**	**1.54**	**-**	**-**

- : no data available

n.r.: not requested by the OECD Guideline

Luminescence values for the negative control wells and the %CV between these values for each run

Negative control	Luminescence reading							Mean	% CV
Run 1	Replicate 1	378866	422119	413546	335618	317716	365676	334680	14.88
	Replicate 2	317060	259389	340164	259248	308682	365781		
	Replicate 3	309392	335692	392675	259223	292587	350804		
Run 2	Replicate 1	346750	576146	588164	507216	532422	544442	472545	15.85
	Replicate 2	444670	438847	468883	465063	542011	470636		
	Replicate 3	527643	507718	355248	363386	383899	442662		

Interpretation of results
GHS criteria not met

Conclusions
Under the experimental conditions of the study, the test item PX-200 was negative in the KeratinoSens assay and therefore was considered to have no potential to activate the Nrf2 transcription factor, though with limitations due to test item precipitation.

3.7.3.3 [Study 3]

Study reference:
Gerbeix, C. 2017

Detailed study summary and results:

Qualifier
according to

Guideline
other: OECD guideline 442E: In vitro skin sensitization: human Cell Line Activation Test (h-CLAT)

Version / remarks
OECD guideline 442E: "In vitro skin sensitization: human Cell Line Activation Test (h-CLAT), 29 July 2016",

Deviations
yes only one dose-range finding assay was performed and only four concentrations were tested.

GLP compliance
no

Type of study
other: human Cell Line Activation Test (h-CLAT)

Test substance

- PX-200, Form: solid: particulate/powder

Details on study design
Test systems: THP-1 cell line.
The THP-1 is an immortalized human monocytic leukemia cell line derived from an acute monocytic leukemia patient. The THP-1 cell line was obtained from ATCC (Ref: TIB-202, American Type Culture Collection, Manassas, USA) by the intermediate of LGC Standards (Molsheim, France).
The THP-1 cells are stored in a cryoprotective medium in a liquid nitrogen container. Cells were grown using general culture procedures. They were cultured in complete RPMI (cRPMI) medium and maintained in a humidified incubator set at 37 °C, 5% CO2. The complete culture medium (cRPM I) was composed of RPMI 1640 with 10% FBS, 0.05 mM 2-mercaptoethanol and with penicillin and streptomycin.

Cell viability was checked using trypan blue.

Vehicle control: Dimethylsulfoxide (DMSO).

This vehicle was used as the vehicle control, and was applied to cells at a concentration of 1% in culture medium.

Negative control: Dimethylsulfoxide (DMSO).

Since the negative control was replaced by a vehicle control, no negative control was used in the present study.

Positive control: 2,4-Dinitrochlorobenzene (DNCB).

DNCB was used formulated in pure DMSO and then diluted in cRPMI medium to reach a final DMSO concentration of 0.2% and a final DNCB concentration of 4 µg/mL.

Solubility assay: The solubility of the test item was assessed visually for each preparation (particles, drops, cloudiness, non-miscible phases, etc). A preparation was deemed appropriate for cell treatment as long as it is

- qualified as a solution or stable dispersion (homogenous emulsion/suspension).

Saline (0.9% NaCl) and DMSO are the only vehicles allowed in the assay. The vehicle was chosen b etween these two in the order of preference, and in accordance with the steps described below.
First, the test item was dissolved in saline at 100 mg/mL. If the test item was soluble or gave a stable dispersion (homogenous emulsion/suspension) saline was used as a vehicle and the highest soluble concentration (HSC) was determined by testing greater concentrations as follows: 300 mg/mL →500 mg/mL.

If not, the test item was dissolved at 500 mg/mL in DMSO. In case it was not soluble at 500 mg/mL in DMSO, the highest soluble concentration (HSC) was determined by testing lower concentrations in a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed). Minimal possible concentration was 1 mg/mL in DMSO.

Test item preparation: On the basis of solubility results, the test item was prepared in the selected vehicle. Sonication for 5 minutes and vortexing for at least 15 minutes were used in order to allow the sol ubilization of the test item.

It was then diluted in treatment culture medium by serial dilutions, using a dilution factor of two (DRF assays) or 1.2 (main tests), to obtain a total of four concentrations.

h-CLAT assay: The study was divided in two successive phases. First, a dose-range finding assay (DRF) was performed to assess test item toxicity and, if applicable, determine the CV75 i.e. the test item concentration that result in 75% cell viability compared to the vehicle control. Secondly, based on cytotoxicity data obtained from the DRF, a concentration series was tested in a minimum of two runs in the main tests to identify potential CD86 and CD54 upregulations.

Results and discussion

Key result
true

Parameter
other:

Vehicle controls valid
yes

Negative controls valid
not applicable

Positive controls valid
yes

Remarks on result
no indication of skin sensitisation

Solubility assay:

Vehicle	Concentration (mg/mL)	Aspect	*Retained vehicle and maximum stock concentration*
0.9% NaCl	100	Colorless heterogeneous solution with white particles *	No
DMSO	500	Whitish heterogeneous suspension with white particles *	No
	250	Heterogeneous suspension	No
	125	Limpid solution after 5 minutes sonication and 5 minutes vortexing	Yes

*: the aspect of these preparations did not change after 5 minutes of sonication.

Based on these data, the selected vehicle was DMSO and the highest concentration used in the main test was 250 µg/mL; *i.e.* the 125 mg/mL stock formulation was diluted 250 times in cRPMI to obtain the working solutions used for treatment. At treatment, a 2-fold dilution was made during the procedure.

Dose-range finding assay:

Dose-range finding results

Study No. 44584 EP

DRF	
Run reference:	20161213
Concentration (µg/mL)	Viability (%)
cRPMI	98.68
0.2% DMSO	98.11
31.25	97.12
62.50	97.71
125.00	96.35
250.00	95.62

CV 75 calculation (DRF1):

Highest concentration (Hc) with viability <75% :	not applicable
Viability value at Hc :	not applicable
Lowest concentration (Lc) with viability >75% :	not applicable
Viability values at Lc :	not applicable
LogCV75 =	not applicable
CV75 (µg/ml) =	not applicable

Summary of the results:

Summary results of all runs and conclusion

Study No. 44584 EP

Test item Name	Conc. (µg/mL)	RFI for CD86 A	RFI for CD86 B	RFI for CD54 A	RFI for CD54 B	Viability (%) A	Viability (%) B	Run conclusion A	Run conclusion B	General conclusion
PX-200	144.68	75	82	122	73	95.5	94.8	N	N	**Negative**
	173.61	63	93	117	82	95.3	95.6			
	208.33	72	81	131	67	95.0	95.2			
	250.00	67	86	117	64	94.5	95.7			

N = run with negative outcome
S = run with positive outcome
I = Invalidated run
Inc = Inconclusive run
Conc. = concentration
RFI = Relative Fluorescence Index

No precipitate/emulsion was noted in the wells following treatment.

Interpretation of results
GHS criteria not met

Conclusions
Under the experimental conditions of this study, the test item PX-200 was negative in the h-CLAT assay.

3.8 Germ cell mutagenicity

Not relevant for this CLH proposal.

3.9 Carcinogenicity

Not relevant for this CLH proposal.

3.10 Reproductive toxicity

Not relevant for this CLH proposal.

3.10.1 Animal data

Not relevant for this CLH proposal.

3.11 Specific target organ toxicity – single exposure

Not relevant for this CLH proposal.

3.12 Specific target organ toxicity – repeated exposure

Not relevant for this CLH proposal.

3.13 Aspiration hazard

Not relevant for this CLH proposal.

4 ENVIRONMENTAL HAZARDS

Not relevant for this CLH proposal.

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