FINAL REPORT

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Study Title

ASSESSMENT OF CONTACT HYPERSENSITIVITY TO TRI-N-OCTYLPHOSPHINE OXIDE IN THE MOUSE (LOCAL LYMPH NODE ASSAY)

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Laboratory Project Identification

Project 507012
Substance 205992/A

1. CONTENTS

1. CONTENTS 2
2. STATEMENT OF GLP COMPLIANCE 3
3. QUALITY ASSURANCE STATEMENT 4
4. SUMMARY 5
5. INTRODUCTION 6
   5.1. Preface 6
   5.2. Aims of the study 6
   5.3. Guidelines 6
   5.4. Storage and retention of records and materials 6
6. MATERIALS AND METHODS 7
   6.1. Test substance 7
      6.1.1. Test substance information 7
      6.1.2. Study specific test substance information 7
      6.1.3. Test substance preparation 7
   6.2. Test system 8
   6.3. Animal husbandry 8
   6.4. Weight of evidence analysis 8
   6.5. Pre-screen test 9
   6.6. Main study 9
      6.6.1. Allocation 9
      6.6.2. Induction - Days 1, 2 and 3 9
      6.6.3. Excision of the nodes - Day 6 10
      6.6.4. Tissue processing for radioactivity - Day 6 10
      6.6.5. Radioactivity measurements - Day 7 10
   6.7. Observations 10
   6.8. Electronic data capture 11
   6.9. Interpretation 11
   6.10. List of deviations 11
      6.10.1. List of protocol deviations 11
      6.10.2. List of standard operating procedures deviations 11
7. RESULTS 12
   7.1. Pre-screen test (Table 1 and Table 2) 12
   7.2. Main study 12
      7.2.1. Skin reactions / Irritation (Table 3) 12
      7.2.2. Systemic toxicity (Body weights, see Table 3) 12
      7.2.3. Macroscopy of the auricular lymph nodes and surrounding area (Table 4) 12
      7.2.4. Radioactivity measurements and SI values (Table 4 and Figure 1) 12
8. CONCLUSION 13
9. REFERENCES 13

TABLES

Table 1: Body weights and skin reactions 14
Table 2: Ear thickness measurements 14
Table 3: Body weights and skin reactions 15
Table 4: Relative size lymph nodes, radioactivity counts (DPM) and Stimulation Index (SI) 16

FIGURES

Figure 1: Dose-response curve 16

APPENDICES

APPENDIX 1: RELIABILITY CHECK

2. STATEMENT OF GLP COMPLIANCE

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with:

The Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) (as revised in 1997) ENV/MC/CHEM (98) 17.

The Sponsor is responsible for all test substance information unless determined by WIL Research Europe B.V..

Analysis of stability, homogeneity and concentration of the test substance under test conditions was not performed as part of this study.

WIL Research Europe B.V.

K. Scase, MSc.
Section Head General Toxicology

Date: 19-December-2014

J.E.H.M. Latour, MSc.
Study Director

Date: 05 January 2015

3. QUALITY ASSURANCE STATEMENT

WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands

This report was inspected by the WIL Research Europe Quality Assurance Unit to confirm that the methods and results accurately and completely reflect the raw data.

During the on-site process inspections procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project 507012

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date
Study	Protocol	07-Oct-2014	07-Oct-2014	07-Oct-2014
	Report	15-Dec-2014	16-Dec-2014	18-Dec-2014
Process	**Analytical and physical chemistry** Test Substance Handling Exposure Observations/Measurements Specimen Handling	05-Aug-2014	12-Aug-2014	13-Aug-2014
	Animal Facilities Test Substance Handling Exposure Observations/Measurements Specimen Handling	13-Oct-2014	24-Oct-2014	28-Oct-2014
	Test Substance Receipt Test Substance Handling	22-Oct-2014	06-Nov-2014	06-Nov-2014
	Test Substance Formulation Test Substance Handling	22-Oct-2014	06-Nov-2014	06-Nov-2014

The review of the final report was completed on the date of signing this QA statement.

WIL Research Europe B.V.
Quality Assurance

C. Mitchell B.Sc., FRQA
Head of Quality Assurance

Date: 19 dec 2014

4. SUMMARY

Assessment of Contact Hypersensitivity to Tri-n-octylphosphine oxide in the Mouse (Local Lymph Node Assay).

The study was carried out based on the guidelines described in:
OECD, Section 4, Health Effects, No.429 (2010),
EC, No 440/2008; B42: "Skin Sensitization: Local Lymph Node Assay"
EPA, OPPTS 870.2600 (2003) "Skin Sensitization".

Test substance concentrations selected for the main study were based on the results of a pre-screen test.

In the main study, three experimental groups of five female CBA/J mice were treated with test substance concentrations of 5, 10 or 25% w/w on three consecutive days, by open application on the ears. Five vehicle control animals were similarly treated, but with vehicle alone (Methyl ethyl ketone).

Three days after the last exposure, all animals were injected with ^{3}H-methyl thymidine and after five hours the draining (auricular) lymph nodes were excised and pooled for each animal.
After precipitating the DNA of the lymph node cells, radioactivity measurements were performed. The activity was expressed as the number of Disintegrations Per Minute (DPM) and a stimulation index (SI) was subsequently calculated for each group.

No irritation of the ears was observed in any of the animals examined. Scaliness was noted for all animals treated at 10% and 25% on Day 6. Thickened ears were noted for all animals treated at 10% and 25% on Day 6.

All auricular lymph nodes of the animals of the experimental groups were considered enlarged.
No macroscopic abnormalities of the surrounding area were noted for any of the animals.

Mean DPM/animal values for the experimental groups treated with test substance concentrations 5, 10 and 25% were 3481, 6207 and 7069 DPM, respectively. The mean DPM/animal value for the vehicle control group was 575 DPM. The SI values calculated for the substance concentrations 5, 10 and 25% were 6.1, 10.8 and 12.3, respectively.

These results show that the test substance elicits a SI ≥ 3. The data showed a dose-response and the EC3 value (the estimated test substance concentration that will give a SI =3) was established to be between 0 and 5%.

The six-month reliability check with Alpha-hexylcinnamaldehyde indicates that the Local Lymph Node Assay as performed at WIL Research Europe is an appropriate model for testing for contact hypersensitivity.

Based on these results:
- according to the recommendations made in the test guidelines (including all amendments), Tri-n-octylphosphine oxide would be regarded as skin sensitizer.

- according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2011) (including all amendments), Tri-n-octylphosphine oxide should be classified as skin sensitizer (Category 1).

- according to the Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (including all amendments), Tri-n-octylphosphine oxide should be classified as skin sensitizer (Category 1) and labeled as H317: May cause an allergic skin reaction.

5. INTRODUCTION

5.1. Preface

Sponsor	Cytec Industries Inc. 5 Garret Mountain Plaza WOODLAND PARK NJ 07424 USA
Study Monitor	Ms. P.A. Vernon
Test Facility	WIL Research Europe B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	J.E.H.M. Latour, MSc.
Study Plan (In life phase)	Start : 29 October 2014 Completion : 24 November 2014

5.2. Aims of the study

The purpose of this study was to evaluate whether the test substance induces contact hypersensitivity in mice after three epidermal induction exposures of the animals under the conditions described in this report. This study should provide a rational basis for risk assessment in man. Compared to sensitization tests using Guinea pigs, the Local Lymph Node Assay (LLNA) provides certain advantages with regard to animal welfare and scientific aspects.

5.3. Guidelines

As required by the Dutch Act on Animal Experimentation (February 1997), this type of protocol was reviewed and agreed by the Laboratory Animal Welfare Officer and the Ethical Committee (DEC 14-23) of WIL Research Europe. The study procedures described in this report were based on the following guidelines:

Organization for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No.429, "Skin Sensitization: Local Lymph Node Assay", Paris Cedex, July 2010.

Commission Regulation (EC) No 440/2008 Part B: Methods for the Determination of Toxicity and other Health Effects; B42: "Skin Sensitization: Local Lymph Node Assay". Official Journal of the European Union No. L142, May 2008, including most recent amendments.

Environmental Protection Agency (EPA): Health Effects Test Guidelines OPPTS 870.2600. "Skin Sensitization", March 2003.

5.4. Storage and retention of records and materials

Records and materials pertaining to the study including protocol, raw data and the final report are retained in the WIL Research Europe archives for a period of at least 2 years after finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. WIL Research Europe will retain information concerning decisions made.

WIL Research Europe will retain a test substance sample until the expiry date, but no longer than 10 years after finalization of the report. After this period the sample will be destroyed.

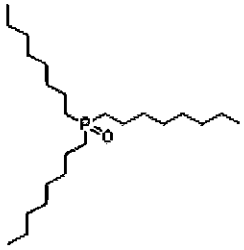
6. MATERIALS AND METHODS

6.1. Test substance

6.1.1. Test substance information

Identification	Tri-n-octylphosphine oxide
Appearance	White solid candle
Batch	Lot#WE8091351
Purity/Composition	96.2%
Test substance storage	At room temperature
Stable under storage conditions until	15 September 2015 (expiry date)

6.1.2. Study specific test substance information

Chemical name (IUPAC), synonym or trade name	Phosphine oxide, trioctyl-; Tri-n-octylphosphine oxide; CYANEX® 921 Extractant; Trioctyl-λ^5-phosphanone; TOPO
CAS Number	78-50-2
Molecular structure	
Molecular formula	$C_{24}H_{51}OP$
Molecular weight	386.64
Irritant or corrosive	Yes
pH (1% in water, indicative range)	4.3 – 3.8 (determined by WIL Research Europe)
Solubility in vehicle:	
• Methylethylketone	Not indicated
Stability in vehicle:	
• Methylethylketone	Not indicated

Note: the pH was determined 1 minute (4.3) after preparing a concentration of 1% test substance in water and at t= 2 hours (3.8).

6.1.3. Test substance preparation

Vehicle	Methyl ethyl ketone (Merck, Darmstadt, Germany).
Rationale	The vehicle was selected on the basis of maximizing the solubility using the test substance data provided by the sponsor and trial formulation results performed at WIL Research Europe. The vehicle was chosen from the vehicles specified in the test guideline: Acetone/Olive oil (4:1 v/v), N,N-dimethylformamide, methylethylketone, propylene glycol and dimethylsulfoxide.
Preparation	The test substance formulations (w/w) were prepared within 4 hours prior to each dosing. No adjustment was made for specific gravity of the vehicle. Homogeneity was obtained to visually acceptable levels.

Correction of the purity/composition of the test substance is not applicable, since the test method requires a logical concentration range rather than specific dose levels to be dosed.

6.2. Test system

Species	Mouse, CBA/J strain, inbred, SPF-Quality. Recognized by the international guidelines as the recommended test system (e.g. OECD, EC, EPA). Source: Janvier, Le Genest-Saint-Isle, France
Number of animals	20 females (nulliparous and non-pregnant), five females per group (main study only).
Age and body weight	Young adult animals (approx. 10 weeks old) were selected. Body weight variation was within +/- 20% of the sex mean.
Identification	Tail mark with marker pen.
Health inspection	At least prior to dosing. It was ensured that the animals were healthy and that the ears were intact and free from any abnormality.
Reliability check	The results of a reliability test with three concentrations of Hexylcinnamaldehyde (CAS Nr. 101-86-0) in Acetone/Olive oil (4:1 v/v), performed not more than 6 months previously and using the same materials, animal supplier, animal strain and essential procedures are summarized in APPENDIX 1 of this report. For both scientific and animal welfare reasons, no concurrent positive control group was added to the study. An extensive data base is available with reliability checks performed each half year during at least the recent 9 years showing reproducible and consistent positive results.

6.3. Animal husbandry

Conditions
Environmental controls for the animal room were set to maintain 18 to 24°C, a relative humidity of 40 to 70%, at least 10 air changes/hour, and a 12-hour light/12-hour dark cycle. Any variations to these conditions were maintained in the raw data and had no effect on the outcome of the study.

Accommodation
Animals were group housed in labeled Makrolon cages (MIII type; height 18 cm) containing sterilised sawdust as bedding material (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany). Paper (Enviro-dri, Wm. Lillico & Son (Wonham Mill Ltd), Surrey, United Kingdom) and shelters (disposable paper corner home, MCORN 404, Datesand Ltd, USA) were supplied as cage-enrichment. The acclimatization period was at least 5 days before the start of treatment under laboratory conditions. On Day 6, the animals were group housed in Makrolon MII type cages with a sheet of paper instead of sawdust and cage enrichment.

Diet
Free access to pelleted rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany).

Water
Free access to tap water.

Diet, water, bedding and cage enrichment evaluations for contaminants and/or nutrients were performed according to facility standard procedures. There were no findings that could interfere with the study.

6.4. Weight of evidence analysis

In the interest of animal welfare and to minimize any testing likely to produce severe responses in animals, a weight of evidence analysis was performed prior to start of this study. All available information was evaluated (e.g. existing human and animal data, literature, substance data supplied by the sponsor, analysis of structure activity relationships (SAR), physicochemical properties and reactivity (pH, buffering capacity)). It was concluded by the Study Director that no severe effects were to be expected.

6.5. Pre-screen test

A pre-screen test was conducted in order to select the highest test substance concentration to be used in the main study. In principle, this highest concentration should cause no systemic toxicity, may give well-defined irritation at most (maximum grade 2 (see section 6.7) and/or an increase in ear thickness < 25%) and is the highest possible concentration that can technically be applied.

Since the test material was expected to be irritating the first animals of the pre-screen test were treated at 5% and 10%. Based on the results of the initially treated animals, four additional animals were treated in a similar manner with two higher concentrations (25% and 50%) at a later stage.

The test system, procedures and techniques were identical to those used in the main study except that the animals were approximately 10-12 weeks (at initiation of treatment) and that the assessment of lymph node proliferation and necropsy were not performed. Two young adult animals per concentration were selected. Each animal was treated with one concentration on three consecutive days. Animals were group housed in labeled Makrolon cages (MII type, height 14 cm). Ear thickness measurements were conducted using a digital thickness gauge (Kroeplin C110T-K) prior to dosing on Days 1 and 3, and on Day 6.

Animals were sacrificed after the final observation.

6.6. Main study

Three groups of five animals were treated with one test substance concentration per group. The highest test substance concentration was selected from the pre-screen test. One group of five animals was treated with vehicle.

6.6.1. Allocation

Group[1]	animal numbers	induction (test substance; % w/w)
1	01 - 05	0 (Methyl ethyl ketone)
2	06 - 10	5
3	11 - 15	10
4	16 - 20	25

[1]. five females each group

6.6.2. Induction - Days 1, 2 and 3

The dorsal surface of both ears was topically treated (25 µL/ear) with the test substance concentration, at approximately the same time on each day. The concentrations were stirred with a magnetic stirrer immediately prior to dosing.

The control animals were treated in the same way as the experimental animals, except that the vehicle was administered instead of the test substance.

6.6.3. Excision of the nodes - Day 6

Each animal was injected via the tail vein with 0.25 mL of sterile phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) containing 20 µCi of ^{3}H-methyl thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA, US).

After five hours, all animals were killed by intraperitoneal injection (0.2 mL/animal) of Euthasol® 20% (AST Farma BV, Oudewater, The Netherlands). The draining (auricular) lymph node of each ear was excised. The relative size of the nodes (as compared to normal) was estimated by visual examination and abnormalities of the nodes and surrounding area were recorded. The nodes were pooled for each animal in approximately 3 mL PBS.

6.6.4. Tissue processing for radioactivity - Day 6

Following excision of the nodes, a single cell suspension of lymph node cells (LNC) was prepared in PBS by gentle separation through stainless steel gauze (diameter 125 µm). LNC were washed twice with an excess of PBS by centrifugation at 200*g* for 10 minutes at 4°C. To precipitate the DNA, the LNC were exposed to 5% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) and stored in the refrigerator until the next day.

6.6.5. Radioactivity measurements - Day 7

Precipitates were recovered by centrifugation, resuspended in 1 mL TCA and transferred to 10 mL of Ultima Gold cocktail (PerkinElmer Life and Analytical Sciences, Boston, MA, US) as the scintillation fluid. Radioactive measurements were performed using a Packard scintillation counter (2800TR). Counting time was to a statistical precision of ± 0.2% or a maximum of 5 minutes whichever came first. The scintillation counter was programmed to automatically subtract background and convert Counts Per Minute (CPM) to Disintegrations Per Minute (DPM).

6.7. Observations

Mortality/Viability	Twice daily.
Body weights	On Day 1 (pre-dose) and Day 6 (prior to necropsy).
Clinical signs	Once daily on Days 1-6 (on Days 1-3 between 3 and 4 hours after dosing).
Irritation	Once daily on Days 1-6 (on Days 1-3 within 1 hour after dosing) according to the following numerical scoring system. Furthermore, a description of all other (local) effects was recorded.

Grading Irritation Reactions:

Erythema and eschar formation:

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

Necropsy	No necropsy for gross macroscopic examination was performed according to protocol.

6.8. Electronic data capture

Observations/measurements in the study were recorded electronically using the following programs: REES Centron Environmental Monitoring system version SQL 2.0 (REES scientific, Trenton, NJ, USA); Quantasmart 2.03 (PerkinElmer Life Sciences, Boston, MA, USA): LSC software.

6.9. Interpretation

DPM values are presented for each animal and for each dose group. A Stimulation Index (SI) is calculated for each group using the individual SI values. The individual SI is the ratio of the DPM/animal compared to DPM/vehicle control group.

If the results indicate a SI ≥ 3, the test substance may be regarded as a skin sensitizer.

The results were evaluated according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2011) (including all amendments) and the Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, including all amendments.

Consideration was given to the EC3 value (the estimated test substance concentration that will give a SI =3) (Ref. 1).

6.10. List of deviations

6.10.1. List of protocol deviations

There were no deviations from the protocol.

6.10.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

7. RESULTS

7.1. Pre-screen test (Table 1 and Table 2)

No signs of systemic toxicity were observed in any of the animals examined. No erythema was observed in any of the animals examined except for the very slight erythema noted for both animals treated at 25% and 50% on Day 6. Scaliness was noted for all animals on Day 6. White staining of the dorsal surface of the ears by test substance remnants was noted for both animals treated at 25% and 50% between Days 1 and 3. The staining did not hamper the scoring of the ears. Thickened ears were noted for the animals treated at 25% between Days 4 and 6.
Variations in ear thickness during the observation period were less than 25% from Day 1 pre-dose values for the animals treated at 5%, 10% and 25% but more than 25% from Day 1 pre-dose values for the animals treated at 50%. Based on these results, the highest test substance concentration selected for the main study was a 25% concentration.

7.2. Main study

7.2.1. Skin reactions / Irritation (Table 3)

No irritation of the ears was observed in any of the animals examined. Scaliness was noted for all animals treated at 10% and 25% on Day 6. Thickened ears were noted for all animals treated at 10% and 25% on Day 6. White staining of the dorsal surface of the ears by test substance remnants was noted for all animals treated at 25% on Days 1, 2 and 3. The staining did not hamper the scoring of the ears.

7.2.2. Systemic toxicity (Body weights, see Table 3)

No mortality occurred and no clinical signs of systemic toxicity were observed in the animals of the main study. Body weights and body weight gain of experimental animals remained in the same range as controls over the study period.

7.2.3. Macroscopy of the auricular lymph nodes and surrounding area (Table 4)

All auricular lymph nodes of the animals of the experimental groups were considered enlarged.
No macroscopic abnormalities of the surrounding area were noted for any of the animals.

7.2.4. Radioactivity measurements and SI values (Table 4 and Figure 1)

Mean DPM/animal values for the experimental groups treated with test substance concentrations 5, 10 and 25% were 3481, 6207 and 7069 DPM, respectively. The mean DPM/animal value for the vehicle control group was 575 DPM. The SI values calculated for the substance concentrations 5, 10 and 25% were 6.1, 10.8 and 12.3, respectively.

8. CONCLUSION

These results show that the test substance elicits a SI ≥ 3. The data showed a dose-response and the EC3 value (the estimated test substance concentration that will give a SI =3) was established to be between 0 and 5%.

The six-month reliability check with Alpha-hexylcinnamaldehyde indicates that the Local Lymph Node Assay as performed at WIL Research Europe is an appropriate model for testing for contact hypersensitivity (see APPENDIX 1).

Based on these results:

- according to the recommendations made in the test guidelines (including all amendments), Tri-n-octylphosphine oxide would be regarded as skin sensitizer.

- according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) of the United Nations (2011) (including all amendments), Tri-n-octylphosphine oxide should be classified as skin sensitizer (Category 1).

- according to the Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (including all amendments), Tri-n-octylphosphine oxide should be classified as skin sensitizer (Category 1) and labeled as H317: May cause an allergic skin reaction.

9. REFERENCES

Ref. 1 Basketter DA, Lea LJ, Dickens A, Briggs, D, Pate I, Dearman RJ and Kimber I. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. J Appl Toxicol 1999;19:261-266.

PRE-SCREEN TEST

Table 1: Body weights and skin reactions

TS[1] (%)	animal	Day 1			Day 2		Day 3		Day 4		Day 5		Day 6		
		bw (g)[2]	erythema[3] left	erythema[3] right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	bw (g)
5	1	25	0	0	0	0	0	0	0	0	0	0	0	0	24
	2	23	0	0	0	0	0	0	0	0	0	0	0	0	24
10	3	21	0	0	0	0	0	0	0	0	0	0	0	0	21
	4	23	0	0	0	0	0	0	0	0	0	0	0	0	24
25	5	22	0	0	0	0	0	0	0	0	0	0	1	1	23
	6	24	0	0	0	0	0	0	0	0	0	0	1	1	25
50	7	24	0	0	0	0	0	0	0	0	0	0	1	1	23
	8	24	0	0	0	0	0	0	0	0	0	0	1	1	24

1. TS = test substance (% w/w).
2. Body weight (grams).
3. Grading erythema and eschar formation (Left = dorsal surface of left ear; right = dorsal surface of right ear):
   0 = No erythema
   1 = Very slight erythema (barely perceptible)

Note: White staining of the dorsal surface of the ears by test substance remnants was noted for both animals treated at 25% and 50% on Days 1, 2 and 3. The staining did not hamper the scoring of the ears. Scaliness was noted for all animals on Day 6. Thickened ears were noted for the animals treated at 25% on Days 4, 5 and 6.

Table 2: Ear thickness measurements

TS[1] (%)	Animal	Day 1		Day 3				Day 6			
		left (mm)	right (mm)	left (mm)	left %[2]	right (mm)	right %[2]	left (mm)	left %[2]	right (mm)	right %[2]
5	1	0.220	0.225	0.220	0	0.220	-2	0.230	5	0.225	0
	2	0.225	0.220	0.220	-2	0.225	2	0.235	4	0.230	5
10	3	0.225	0.225	0.225	0	0.220	-2	0.245	9	0.245	9
	4	0.220	0.225	0.220	0	0.230	2	0.245	11	0.240	7
Additional pre-screen tests:											
25	5	0.225	0.220	0.225	0	0.225	2	0.250	11	0.250	14
	6	0.220	0.220	0.220	0	0.220	0	0.255	16	0.260	18
50	7	0.230	0.225	0.225	-2	0.225	0	0.560	143	0.550	144
	8	0.225	0.225	0.225	0	0.225	0	0.550	144	0.550	144

Left (mm) = thickness of left ear in millimeters; right (mm) = thickness of right ear in millimeters.
1. TS = test substance (% w/w).
2. Percent increase compared to Day 1 pre-dose value.

MAIN STUDY

Table 3: Body weights and skin reactions

group	TS [1] (%)	animal	Day 1			Day 2		Day 3		Day 4		Day 5		Day 6		
			bw (g) [2]	erythema [3] left	erythema [3] right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	erythema left	erythema right	bw (g)
1	0	1	23	0	0	0	0	0	0	0	0	0	0	0	0	24
		2	20	0	0	0	0	0	0	0	0	0	0	0	0	22
		3	22	0	0	0	0	0	0	0	0	0	0	0	0	23
		4	21	0	0	0	0	0	0	0	0	0	0	0	0	22
		5	24	0	0	0	0	0	0	0	0	0	0	0	0	23
2	5	6	21	0	0	0	0	0	0	0	0	0	0	0	0	22
		7	24	0	0	0	0	0	0	0	0	0	0	0	0	25
		8	23	0	0	0	0	0	0	0	0	0	0	0	0	23
		9	22	0	0	0	0	0	0	0	0	0	0	0	0	23
		10	24	0	0	0	0	0	0	0	0	0	0	0	0	23
3	10	11	23	0	0	0	0	0	0	0	0	0	0	0	0	23
		12	24	0	0	0	0	0	0	0	0	0	0	0	0	24
		13	23	0	0	0	0	0	0	0	0	0	0	0	0	23
		14	24	0	0	0	0	0	0	0	0	0	0	0	0	25
		15	22	0	0	0	0	0	0	0	0	0	0	0	0	24
4	25	16	25	0	0	0	0	0	0	0	0	0	0	0	0	23
		17	23	0	0	0	0	0	0	0	0	0	0	0	0	25
		18	23	0	0	0	0	0	0	0	0	0	0	0	0	24
		19	22	0	0	0	0	0	0	0	0	0	0	0	0	23
		20	25	0	0	0	0	0	0	0	0	0	0	0	0	24

[1]. TS = test substance (% w/w).
[2]. Body weight (grams).
[3]. Grading erythema and eschar formation (Left = dorsal surface of left ear; right = dorsal surface of right ear):
0 = No erythema

Note: White staining of the dorsal surface of the ears by test substance remnants was noted for all animals treated at 25% on Days 1, 2 and 3. The staining did not hamper the scoring of the ears. Scaliness was noted for all animals treated at 10% and 25% on Day 6. Thickened ears were noted for all animals treated at 10% and 25% on Day 6.

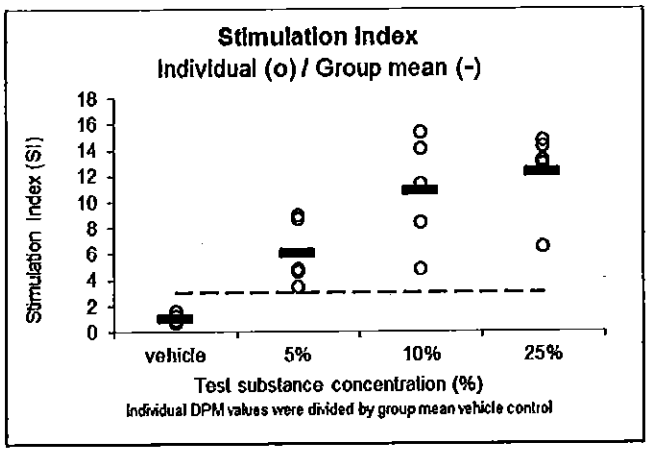
MAIN STUDY

Table 4: Relative size lymph nodes, radioactivity counts (DPM) and Stimulation Index (SI)

group	TS [1] (%)	animal	Size nodes [2] left	Size nodes [2] right	DPM [3] / animal	mean DPM ± SEM [4]	mean SI ± SEM
1	0	1	n	n	469		
		2	n	n	897		
		3	n	n	697	575 ± 99	1.0 ± 0.2
		4	n	n	345		
		5	n	n	468		
2	5	6	+	+	1950		
		7	+	+	5116		
		8	+	+	2730	3481 ± 656	6.1 ± 1.5
		9	+	+	4989		
		10	+	+	2619		
3	10	11	+	+	4840		
		12	+	+	8819		
		13	+	+	8101	6207 ± 1107	10.8 ± 2.7
		14	+	+	6547		
		15	+	+	2728		
4	25	16	+	+	8182		
		17	+	+	7540		
		18	+	+	3733	7069 ± 856	12.3 ± 2.6
		19	+	+	8462		
		20	+	+	7429		

1. TS = test substance (% w/w).
2. Relative size auricular lymph nodes (-, -- or ---: degree of reduction, +,++ or +++: degree of enlargement, n: considered to be normal).
3. DPM = Disintegrations per minute
4. SEM = Standard Error of the Mean

Figure 1: Dose-response curve



APPENDIX 1: RELIABILITY CHECK

ASSESSMENT OF CONTACT HYPERSENSITIVITY

TO Alpha- Hexylcinnamaldehyde, technical grade

IN THE MOUSE (LOCAL LYMPH NODE ASSAY)

A RELIABILITY CHECK.

Project 507606

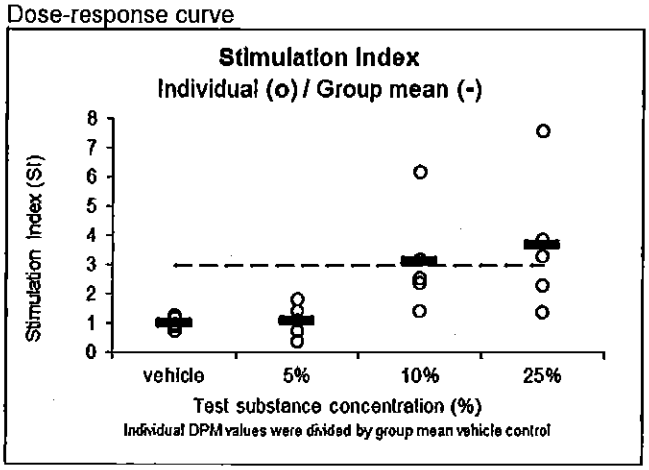
SUMMARY RELIABILITY CHECK

A reliability check is carried out at regular intervals to check the sensitivity of the test system and the reliability of the experimental techniques as used by WIL Research Europe. In this study, performed in November 2014, females of the CBA/J mouse strain (Janvier, Le Genest-Saint-Isle, France) were checked for sensitivity to Hexylcinnamaldehyde. The females were approx. 10 weeks old at commencement of the study. The study was based on the OECD Guideline No. 429, EC No 440/2008, Part B.42 and EPA, OPPTS 870.2600 "Skin Sensitization". Alpha- Hexylcinnamaldehyde, technical grade (CAS no. 101-86-0) was fabricated under lot no. MKBJ8846V (Sigma- Aldrich, Steinheim, Germany). Concentrations used for this study were 5, 10 and 25% in Acetone/Olive oil (4:1 v/v).

Group[1]	% Alpha- Hexylcinnamaldehyde, technical grade	mean DPM ± SEM			mean SI ± SEM		
2	5%	583	±	137	1.1	±	0.3
3	10%	1696	±	438	3.1	±	0.9
4	25%	1994	±	575	3.7	±	1.1
1	0% (Acetone:Olive oil (4:1 v/v))	543	±	51	1.0	±	0.1

[1] Five females per group.

Dose-response curve



CONCLUSION

The SI values calculated for the substance concentrations 5, 10 and 25% were 1.1, 3.1 and 3.7 respectively. An EC3 value of 9.8% was calculated using linear interpolation.

The calculated EC3 value was found to be in the acceptable range of 4.8 and 19.5%. The results of the 6 monthly HCA reliability checks of the recent years were 14.4, 16.5, 14.5, 13.4, 14.1 and 17.3%.

Based on the results, it was concluded that the Local Lymph Node Assay as performed at WIL Research Europe is an appropriate model for testing for contact hypersensitivity.

The raw data, protocol and report from this study are kept in the WIL Research Europe archives. The test described above was performed in accordance with WIL Research Europe Standard Operating Procedures and the report was audited by the QA-unit.