# 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:

# Benzyl salicylate

**EC Number:** **204-262-9**

**CAS Number:** **118-58-1**

**Index Number:** **-**

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# 1 PHYSICAL HAZARDS

Not evaluated for this dossier

# 2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Not evaluated in this dossier which addresses skin sensitisation only. Induction of skin sensitisation takes place locally in the skin at the site of contact, therefore systemic availability of the hapten is not relevant. Proof of sensitisation after dermal contact also proves that a sufficient amount of hapten has been taken up.

# 3 HEALTH HAZARDS

## Acute toxicity

### 3.1 Acute toxicity - oral route

Not evaluated for this dossier

### 3.2 Acute toxicity - dermal route

Not evaluated for this dossier

### 3.3 Acute toxicity - inhalation route

Not evaluated for this dossier

### 3.4 Skin corrosion/irritation

Not evaluated for this dossier

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Not evaluated for this dossier

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Not evaluated for this dossier

## 3.7 Skin sensitisation

### 3.7.1 Animal data

#### 3.7.1.1 Mouse local lymph node assay (LLNA, key study)

**Study reference:**

Central Toxicology Laboratory (2005): Benzyl salicylate diluted with vehicle 1:3 EtOH:DEP: Local lymph node assay. Report no. 47378, study no. GM7852, date: 2005-01-20. CTL Cheshire, United Kingdom. Research Institute for Fragrance Materials, New Jersey, United States, Unpublished study report[1]

**Detailed study summary and results:**

In a study performed according to OECD TG 429/EU B.42 (GLP, reliable without restriction) benzyl salicylate has been demonstrated to be a skin sensitiser. Animals were obtained from Harlan Interfauna Ltd, Blackthorne, Bicester, Oxon, United Kingdom. Female CBA mouse young adults (8 - 12 weeks) were used with the weight at study initiation spanning from 16.8 to 21.1 g. Diet and mains water were available ad libitum. For the experiments, 4 animals per dose were used. The vehicle used in experiments was ethanol:diethyl phthalate (1:3) while the concentrations used were 2.5, 5, 10, 25, and 50% w/v. Hexyl cinnamic aldehyde (CAS No 101-86-0) in acetone:olive oil (4:1) was used as positive control.

Details on the test material: The benzyl salicylate used was a colourless liquid with an analytical purity of 99.8% (purity test date: 2004-11-09; Lot No: 2004134-0010; expiration date of the lot: 2006-05-13) that was stored at ambient temperature in the dark. No data are available on identity and concentrations of impurities, composition of test material, or stability under test conditions.

Approximately 25 µL of preparation of the relevant concentration of test substance/vehicle were applied to the dorsal surface of each ear using a variable volume micropipette. A vehicle control group was similarly treated using vehicle alone. This procedure was repeated daily for 3 consecutive days. Three days after the third application, all the animals were injected, via the tail vein, with 250 µL of phosphate buffered saline (PBS) containing 25 µCi of a 2.0 Ci/mmol specific activity $^{3}$H-methyl thymidine. Approximately 5 hrs later, the animals were humanely killed by inhalation of halothane vapour followed by cervical dislocation. The draining auricular lymph nodes were removed from each animal and, together with the nodes from the other animals in the group, were placed in a container of PBS.

A single cell suspension was prepared by mechanical disintegration of lymph nodes through a 200-mesh stainless steel gauze. The cell suspensions were washed 3 times by centrifugation with approximately 10 mL of PBS. Approximately 3 mL of 5 % w/v trichloroacetic acid (TCA) were added and, after overnight precipitation at 4 °C, the samples were pelleted by centrifugation and the supernatant was discarded. The cells were then re-suspended in approximately 1 mL of TCA. The lymph node suspensions were transferred to scintillation vials and 10 mL of scintillant (Octiphase) was added prior to β-scintillation using a Packard Tri-Car Liquid scintillation counter. Animals were checked at least daily for signs of systemic toxicity. Mention of scoring of ear erythema or ear thickness measurements obtained either by using a thickness gauge, or ear punch weight determinations at necropsy were not done.

The EC3 value was derived by interpolating between two points on the stimulation index axis, using values immediately above and below the value of 3. The equation is as follows: $EC_3 = (3 - d) \times (a - c) \div (b - d) + c$ (a = concentration giving the SI immediately above 3; b = SI value of "a"; c = concentration giving the SI immediately below 3; d = The SI value of "c"). From this value a value of quantity applied per square centimetre was derived, assuming that the area of the mouse ear is 1 cm² and that 1 µL is equivalent to 1 mg.

[1] The results of this study were also utilised in (Api et al., 2015).

Table 1: LLNA results for benzyl salicylate

| Concentration (%) | SI | Disintegrations per minute (DPM) |
|---|---|---|
| 0 | 0 | 2,248 |
| 2.5 | 2.6 | 5,728 |
| 5 | 5.5 | 12,419 |
| 10 | 6 | 13,518 |
| 25 | 18.9 | 42,548 |
| 50 | 26.2 | 58,830 |

The EC3 value was determined to be 2.9%. The quantity applied per square centimetre was 725 µg/cm. The outcome of the positive control testing using hexylcinnamaldehyde confirmed the suitability of the test design. Overall, benzyl salicylate was found to be a skin sensitiser in an LLNA test compliant with OECD TG 429 (Central Toxicology Laboratory, 2005).

### 3.7.1.2 Short-period test (14 days) in guinea pigs

**Study reference:**

Kashima R., Oyake Y., Okada J., and Ikeda Y. (1993): Studies of new short-period method for delayed contact hypersensitivity assay in the guinea pig. (I). Development and comparison with other methods. Contact Dermatitis 28 (4), 235-242. DOI: 10.1111/j.1600-0536.1993.tb03409.x

**Detailed study summary and results:**

Four week-old Female Hartley strain albino guinea pigs (approx.. 250 g, microbial grade, clean) were purchased from Japan SLC, Inc.. Animals were used for the experiment at 6 weeks of age, housed in aluminium cages in groups of 5 animals and fed with solid diet (Labo G standard, Nihon Nousan) and tap water sterilised by UV, available ad libitum. Animal rooms were maintained on a 12- h light/12 h -dark cycle with light on at 7:00 and off at 19:00, at a room temperature of 23 ± 2 °C, a relative humidity of 55 ± 5% and a ventilation of 15 cycles/h. Benzyl salicylate (special grade) (purity not given) was used for sensitisation experiments. Before the main study, the concentration of test chemical causing slight erythema was determined by preliminary primary skin irritation testing.

The sensitisation unit was an occlusive patch consisting of a 2 x 4 cm lint pad (Yabane jirushihonpo, Tokyo, Japan) containing 0.2 mL of test solution attached to 5 x 10 cm surgical tape (Blenderm ™, 3M, St. Paul, USA). This patch was applied to the animals' skin, and then bandaged 3 x with adhesive elastic bandage (Silkytex No. 5, Tokyo Eizai Lab. Co. Ltd, Tokyo, Japan). The challenge unit was an occlusive patch consisting of adhesive plaster for patch tests (Torii Pharmaceutical Co Ltd, Tokyo, Japan) placed on an adhesive sponge (Reston ™, 3M, St. Paul, USA). This unit was divided into 2 sets containing 3 lint patches (approx. 1.5 cm). An occlusive patch containing 0.1 mL of test solution on each of the lint pad was applied to the animals' skin, and then bandaged 2 or 3 x in 3 places with surgical tape (Transpore™, 3M, St. Paul, USA).

The sensitisation studies were carried out in the form of a short-term (subacute) contact hypersensitivity assay. 1st day: an area of 5 x10 cm on the scapular region was clipped with an electric clipper and shaved with an electric shaver. FCA (undiluted) was injected intradermally (i.d.) into the scapular region at a small inner site on each side of the 2 x 4cm area (0.1 mL). A second FCA (undiluted) i.d. injection was administered in the area of the first injection site. An occlusive patch was applied for 24 h to the 2 x4 cm site on the scapular region with the occlusive patch containing 0.2 mL of test solution. The correspondence between the positive rate of the test sample and the assay method was rated by sensitisation experiments, varying the combination and number of FCA (undiluted) i.d. injections and 24 h occlusive patch. Control group animals were only treated with Freund's complete adjuvant FCA (undiluted) i.d. injections. The sensitisation and control groups comprised 10 and 5 animals, respectively. On challenge day, the flank skin was clipped and shaved. The 1st and 2nd challenges were carried out by non-occlusive topical application. Test solution (0.02 mL) was applied to 2.0 cm diameter areas of the flank skin with a silicone stick (5.0 mm diameter, Mitsui Dupont). The 3rd challenge was carried out with a 24 h occlusive patch which contained 0.1 mL of test solution. The 2nd challenge was performed 3 weeks after the 1st challenge and the 3rd challenge 1

week after the 2nd challeng. Before the challenge, the highest concentration of test chemical causing no irritation was determined by preliminary primary skin irritation testing. For this test, animals were treated with 0.1mL of Freund's complete adjuvant FCA (undiluted) i.d. injection into 2 sites in the scapular region 7 days before the test. After non-occlusive topical application challenge, animals were placed individually for 1- 2 h (long enough for drying) in 5 series of cages allowing no interference.

The reaction was evaluated 24, 48, and 72 h after challenge, according to the following criteria and scores in parentheses: -: no reaction (0); ±: slight erythema (1); +: apparent erythema (2); ++: apparent erythema with edema (3); + + +: crust or necrosis (4). A reaction ≥ + was considered to be positive. The short-term AP2 test was compared with the CCET and guinea pig maximisation (GPMT) tests by evaluating the contact hypersensitivity of benzyl salicylate. The results can be found in Table 2 below.

Table 2: Delayed contact hypersensitivity assay in guinea pigs for benzyl salicylate, taken from (Kashima et al., 1993b)

| Method | Challenge conc. % (w/w) | 1st challenge 24 h | 1st challenge 48 h | 1st challenge 72 h | 2nd challenge 24 h | 2nd challenge 48 h | 2nd challenge 72 h | 3rd challenge[c] 24 h | 3rd challenge[c] 48 h | 3rd challenge[c] 72 h |
|---|---|---|---|---|---|---|---|---|---|---|
| AP2[b] | 10.0% | 9/10 (2.0)[a] | 9/10 (2.0) | 7/10 (1.8) | 4/10 (1.4) | 4/10 (1.1) | 2/10 (0.8) | 6/10 (1.4) | 8/10 (1.8) | 8/10 (1.9) |
| | 3.0% | 5/10 (1.4) | 4/10 (1.3) | 3/10 (0.9) | 4/10 (1.0) | 1/10 (0.6) | 1/10 (0.5) | 5/10 (1.2) | 7/10 (1.6) | 7/10 (1.6)[d] |
| | 1.0% | 1/10 (0.8) | 0/10 (0.5) | 0/10 (0.5) | 4/10 (0.9) | 1/10 (0.4) | 1/10 (0.6) | 3/10 (0.9) | 4/10 (1.2) | 5/10 (1.2)[d] |
| CCET[b] | 10.0% | 8/10 (1.8) | 6/10 (1.6) | 2/10 (0.9) | 4/10 (1.1) | 3/10 (1.1) | 1/10 (0.6) | | | |
| | 3.0% | 3/10 (1.1) | 2/10 (0.8) | 0/10 (0.2) | 2/10 (0.9) | 0/10 (0.5) | 0/10 (0.1) | | ——— | |
| | 1.0% | 1/10 (0.6) | 0/10 (0.3) | 0/10 (0.2) | 1/10 (0.7) | 0/10 (0.5) | 0/10 (0.2) | | | |
| GPMT[c] | 0.03% | 0/10 (0.8) | 2/10 (1.0) | 3/10 (0.9) | 2/10 (0.9) | 3/10 (1.0)[d] | 3/10 (0.7)[d] | | | |
| | 0.01% | 0/10 (0.7) | 2/10 (0.7) | 2/10 (0.8) | 0/10 (0.7) | 2/10 (0.9)[d] | 2/10 (0.6)[d] | | ——— | |
| | 0.003% | 0/10 (0.5) | 3/10 (0.7) | 3/10 (0.6) | 0/10 (0.7) | 2/10 (0.8) | 3/10 (0.8)[d] | | | |

[a] Postive number/total number, and mean response (score) in parenthesis.
[b] The 1st and the 2nd challenges in the AP2 and CCET were carried out by non-occlusive topical application with 0.02 ml of 10% (w/w), 3% and 1% in ethanol.
[c] The 1st and the 2nd challenges in the GPMT, and the 3rd challenge in the AP2, were carried out by 24-h occlusive patch with 0.1 ml of 0.03% (w/w), 0.01% and 0.003% in ethanol.
[d] Positive skin reaction was observed in a few control group animals.
Induction concentrations were 10% (w/w) in liquid paraffin for injection (GPMT), and 30% (w/w) in ethanol for 24-h (AP2, CCET) and 48-h (GPMT) occlusive patch.

The results from all four studies demonstrate a sensitising effect of benzyl salicylate (Kashima et al., 1993b).

### 3.7.1.3 Enhancement effect of cyclophosphamide on delayed contact hypersensitivity

**Study reference:**

Kashima R., Oyake Y., Okada J., and Ikeda Y. (1993): Studies of new short-period method for delayed contact hypersensitivity assay in the guinea pig. (2). Studies of the enhancement effect of cyclophosphamide. Contact Dermatitis 29 (1), 26-32. DOI: 10.1111/j.1600-0536.1993.tb04532.x

**Detailed study summary and results:**

To achieve an enhancement effect on the detection of sensitivity in the contact hypersensitivity assay in Hartley guinea pigs, the authors of the study described in the previous section used cyclophosphamide for immune potentiation due to its influence on suppressor lymphocytes (B cells and suppressor T cells). Cyclophosphamide was dissolved in physiological saline just prior to the experiments and administered intraperitoneally (i.p.). A combination of adjuvant injection and 24 h occlusive patch sensitisation was performed on days 3 and 7 after cyclophosphamide administration. This method was designated as the "CAP2" test. By modifing the AP2 test, the correspondence of the positive rates of benzyl alcohol to assay methods was examined. Combinations of cyclophosphamide i.p. administration with the number of times of FCA (undiluted) intradermal (i.d.) injection and 24-h occlusive patch sensitisation were varied. Control group animals were only treated with FCA (undiluted) i.d. injection and cyclophosphamide i.p. administration. The experimental group consisted of 10, and the control group of 5 animals.

The results of this study are listed in Table 3. When compared with the results in (Kashima et al., 1993b), the CAP2 test gave the highest positive rate at the 1st challenge; it was higher than the GPMT and the AP2 test at the 2nd and 3rd challenges (Kashima et al., 1993a).

Table 3: The enhancement effect of cyclophosphamide (CY) in the CAP2 test on delayed contact hypersensitivity caused by a topical induction concentration of 30% (w/w) benzyl salicylate (Kashima et al., 1993a)*

| Challenge concentrations in % (w/w) | Time-point (h) | 1st challenge: | 2nd challenge: | 3rd challenge |
|---|---|---|---|---|
| 1st and 2nd challenge: 10.0<br>3rd challenge: 0.03 | 24 | 100 (2.9) | 90 (2.5) | 90 (2.2) |
| | 48 | 100 (2.7) | 80 (2.2) | 90 (2.5) |
| | 72 | 100 (2.8) | 50 (1.5) | 100 (2.7) |
| 1st and 2nd challenge: 3.0<br>3rd challenge: 0.01 | 24 | 90 (2.6) | 90 (2.4) | 70 (1.9) |
| | 48 | 90 (2.5) | 80 (1.8) | 100 (2.5) |
| | 72 | 90 (2.3) | 20 (1.1) | 90 (2.5) |
| 1st challenge: 1.0<br>2nd challenge: 0.003 | 24 | 90 (2.1) | 90 (2.0) | 40 (1.5) |
| | 48 | 90 (2.0) | 80 (1.7) | 70 (1.9) |
| | 72 | 90 (2.0) | 10 (1.0) | 70 (1.7) |

* data are shown as positive/total number (in %) and mean response (score) in parenthesis, n = 10 in all groups

### 3.7.1.4 Cumulative contact enhancement test (CCET, supporting study)

**Study reference:**

Imokawa G. and Kawai M. (1987): Differential hypermelanosis induced by allergic contact dermatitis. Journal of Investigative Dermatology 89 (6), 540-546. DOI: 10.1111/1523-1747.ep12461181

**Detailed study summary and results:**

In this study the authors addressed the issue of hyperpigmentation caused by certain allergens and demonstrated that allergic contact dermatitis can stimulate epidermal pigment cell function in an allergen-dependent manner. Benzyl salicylate was used for a cumulative contact enhancement test (CCET) according to the protocol developed by (Tsuchiya et al., 1982). An area on the back of tortoise shell guinea pigs (weight 250- 300 g) was shaved with electric clippers and then with an electric razor. For sensitisation, a 24 h closed patch test using 100% benzyl salicylate was performed in 10 animals every third day for 2 weeks (with a maximum of 4 applications). An injection of Freund's complete adjuvant (FCA) was intradermally (i.d.) administered before the third closed patch test. An untreated group of 5 animals was used as control. Eleven days after the last patch test, challenge procedures were performed daily for 1-3 days by applying 0.01 mL of ethanol solution containing 50% benzyl salicylate to 3 non-treated areas on the back of the guinea pigs. The reaction was evaluated over a period of 43 days using the following skin reaction scale: Erythema: - (0) no reaction, ± (0.5) slight erythema, + (1.0) apparent erythema, ++ (2.0) moderate erythema with edema. Pigmentation: - (0) no pigmentation, ± (0.5) minimal visible pigmentation, + (1.0) moderate pigmentation, ++ (2.0) intense deep pigmentation. Calculation of the skin reaction was performed by averaging total scores from 30 reaction areas (10 animals, each animal possessing 3 separate treated areas).

Table 4: Cumulative contact enhancement test (CCET) (Imokawa and Kawai, 1987)

| Intensity of reaction | - | ± | + | ++ |
|---|---|---|---|---|
| **Fraction of animals affected by an allergic reaction 24 h after the last application (%)** | 37 | 20 | 33 | 10 |
| **Fraction of animals affected by pigmentation on day 25 after the last application (%)** | 90 | 10 | 0 | 0 |

The results show that benzyl salicylate triggered positive reactions upon scoring following intense allergic reactions after the challenge application (Imokawa and Kawai, 1987).

### 3.7.1.5 Standard and modified maximisation test

**Study reference:**

Maurer T. and Hess R. (1989): The maximisation test for skin sensitization potential - updating the standard protocol and validation of a modified protocol. Food and Chemical Toxicology 27 (12), 807-811. DOI: 10.1016/0278-6915(89)90112-9

**Detailed study summary and results:**

All the tests were performed with guinea pigs of the Pirbright White strain (at least five males and five females in each group), bred on the authors' premises (Tif: DHP) and weighing between 320 and 400 g. The animals were identified with individual ear tags and housed individually in Macrolon cages (Type 3) at a constant room temperature of 21 ± 2 °C, with a relative humidity of 50 ± 10%, and a 12 h light/12 h dark cycle. They received diet vitamin C -enriched, guinea-pig pellets (Nafag No. 846, Gossau, St. Gallen, Switzerland) and water ad libitum. The diet was supplemented daily with fresh carrots. The animals were randomly distributed to the test groups and were acclimatised for one week before starting the experiments. Benzyl salicylate was obtained from Jules Chiquet, Basel, Switzerland (purity not mentioned).
The standard maximisation test was performed with an initial induction with two intradermal injections into the skin of the animals' neck (0.1 mL of each of the test compounds in saline solution). The test compound incorporated in an adjuvant-saline mixture, and the adjuvant-saline mixture alone were applied (a total of six injections).The adjuvant-saline mixture was freshly made by adding the saline dropwise to the adjuvant. One week later the test compound was incorporated in soft white petrolatum and applied to the injection sites on the animals' neck percutaneously under occlusion for 48 h. If the test compound had no primary irritant effect, the application site was pre-treated epidermally with 10% sodium lauryl sulphate in soft white petrolatum without occlusion. Two weeks after epidermal induction, the animals were tested on one flank with the maximal sub-irritant concentration of the test compound in soft white petrolatum (24 h occlusive administration).

For the modified maximisation test, the initial induction consisted of four intradermal injections (0.1 mL each) of an adjuvant-saline mixture into the skin of the animals' neck, followed by application of the test compound incorporated in soft white petrolatum, on the injection sites under occlusion for 24 h. The remaining treatment was as described for the standard maximisation test. Control groups were treated with the vehicles alone during the induction. The control animals were challenged with the vehicle and the test compounds at maximal sub-irritant concentrations.

Alternative protocols were used for comparison: the modified protocol of (Sato et al., 1981) and the cumulative contact enhancement test (CCET) of (Tsuchiya et al., 1982). The induction was conducted according to the original publications. The challenge was performed in all groups with vaseline as vehicle and 24 h occlusive application while in the CCET method the first challenge was by open epidermal applications. The reactions after epidermal challenge application were assessed according to the scoring system for primary irritation 24 and 48 h after removing the bandages. Approximately 3 h before the first evaluation the skin was chemically depilated.

Benzyl salicylate tested positive in the modified GPMT, i.e. 40-50/100% of the animals showed a positive response upon the first/second challenge (Maurer and Hess, 1989).

### 3.7.1.6 Modified Guinea Pig Maximisation Test (GPMT)

**Study reference:**

Hausen B.M. and Wollenweber E. (1988): Propolis allergy. (III). Sensitization studies with minor constituents. Contact Dermatitis 19 (4), 296-303. DOI: 10.1111/j.1600-0536.1988.tb02931.x

**Detailed study summary and results:**

This study was performed with female Pirbright White guinea pigs, n = 10/group, weight: 280-350 g, housed 3/cage in a room with a temperature of 22-25 °C, humidity of 50-55%, artificial illumination for 10 h/d, which were provided with Altromin® diet and water ad libitum. Readings were performed every day at the same time under the same light conditons. Benzyl salicylate was obtained from Haarmann & Reimer, Holzminden. Sensitisation was performed with 6 i.d. injectins of 0.1-0.15 mL of emulsion (15 mg ester in 4 mL FCA and emulsified in 4 mL physilogical saline) in a semi-circular arc in the clipped and shaved shoulder area. The procedure was repeated on the $5^{th}$ and on the $9^{th}$ day, leaving a gap of 2-3 cm between the rows. Each animal received in total 4.5 mg of the substance. The threshold of iritation was determined in 10 guinea pigs, treated with an emulsion of 4 mL FCA and 4 mL physiological saline, but without the test material. The open epicutaneous application of 3 dilutions (10, 3, and 1% in acetone) was performed, and read 24 h later. Challenge was performed 11 days after induction, open epicutaneous elicitation was

performed by application of 0.05 mL of sub-irritant doses and further dilutions (all in acetone) onto the clipped and shaved right flanks of the sensitised animals. The reactions were read at 24, 48, and 72 h. The mean response was computed as: sum of all observed reactions/total number of treated animals.

Benzyl salicylate was tested positive in the modified Guinea Pig Maximisation Test (GPMT), as seen in Table 5 below (Hausen and Wollenweber, 1988).

Table 5: The results of the sensitisation expenments for benzyl salicylate.

| Time (h) | +++ | ++ | + | (+) | 0 | Mean response |
|---|---|---|---|---|---|---|
| **Concentration 1%** | | | | | | |
| **24** | 0 | 5 | 2 | 3 | 0 | 1.35 |
| **48** | 0 | 5 | 2 | 3 | 0 | 1.35 |
| **72** | 1 | 3 | 2 | 2 | 2 | 1.2 |
| **Concentration 0.1%** | | | | | | |
| **24** | 0 | 5 | 1 | 2 | 2 | 1.2 |
| **48** | 0 | 2 | 2 | 4 | 2 | 0.8 |
| **72** | 0 | 2 | 3 | 2 | 3 | 0.8 |

### 3.7.1.7 Reviews[2] citing[3] further animal data

**Study references:**

Belsito D., Bickers D., Bruze M., Calow P., Greim H., Hanifin J.M., Rogers A.E., Saurat J.H., Sipes I.G., and Tagami H. (2007): A toxicologic and dermatologic assessment of salicylates when used as fragrance ingredients. Food and Chemical Toxicology 45 (1 SUPPL.), S318-S361. DOI: 10.1016/j.fct.2007.09.066

Lapczynski A., McGinty D., Jones L., Bhatia S., Letizia C.S., and Api A.M. (2007): Fragrance material review on benzyl salicylate. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 45 Suppl 1, S362-380. DOI: 10.1016/j.fct.2007.09.036

**Detailed study summary and results:**

For a tabular overview, cf. Table 9 of the main study. Full references have not been included in the reference list of this dossier, but can be accessed via the reference lists in the above two reviews. Paragraph numbers used below refer to the study numbers given there.

1. A guinea pig maximisation test was conducted in 8 test and 8 control female albino Hartley-Dunkin guinea pigs weighing 435–490 g. Induction consisted of a two-stage procedure. In the first stage, three intradermal injections (0.1 mL each) were administered to the clipped shoulder region of each animal. The injections consisted of Freund's Complete Adjuvant (FCA) plus distilled water (1:1), 10% w/v benzyl salicylate in FCA, and finally 10% w/v benzyl salicylate in FCA plus distilled water (1:1). The second stage was a 48-h topical application made seven days later to the same area on the shoulder. The shoulder was shaved again and treated with 10% sodium lauryl sulfate (SLS) in petrolatum. Two weeks after the topical application, the flank of each animal was shaved free of hair and divided into three sites (1.5 cm x 1.5 cm). The challenge test was performed by applying 0.02 mL of 5, 10, and 20% benzyl salicylate in acetone to each site. The application sites were left uncovered. Reactions were graded using the Draize scale 24, 48, and 72 h after application. Sensitisation was observed at all concentrations (RIFM, 1997c).

2. A guinea pig maximisation test was conducted using benzyl salicylate at 10% for both induction and challenge phase. Sensitisation was observed (Ishihara et al., 1986).

3. A Magnusson–Kligman guinea pig maximisation test was conducted in 10 Hartley guinea pigs/dose using 1% benzyl salicylate in ethanol intradermally and 100% dermally. No sensitisation reactions were observed (Tsuchiya et al., 1982).

[2] Since the two reviews mostly address the same studies, they are reported jointly.

[3] With the exception of the studies mentioned elsewhere in the main dossier or this annex, the references cited in these reviews have not been accessed directly by the DS.

4. Four week old female Hartley strain guinea pigs (20/group) weighing about 300 g were tested in a guinea pig maximisation test. Benzyl salicylate at 10% in liquid paraffin and FCA was intradermally injected in the shoulder region of each animal. Five days after the intradermal injections 10% SLS in petrolatum was topically applied to the same region. Twenty-four hours later, 50% benzyl salicylate in white petrolatum was applied for 48 h with impermeable tape and adhesive bandage. Two weeks after the topical application, benzyl salicylate at 5, 10, and 20% in white petrolatum was applied to the backs of the animals using mini-plasters (Torii Pharmaceutical Co.) for 24 h. The reactions were read 24 and 48 h after removal of the plaster. Two reactions were observed at 20%. Questionable reactions were observed in three (3/20) animals at 5%, five (5/20) animals at 10%, and four (4/20) animals at 20% (Kozuka et al., 1996).

5. A guinea pig maximisation test was conducted using outbred Himalayan White-Spotted male and female guinea pigs. Induction was via two intradermal injections of 0.1 mL of 5% benzyl salicylate in white petrolatum with and without FCA on day 0. On day 8, 25% benzyl salicylate in petrolatum was applied to a clipped area on the neck for 48 h under occlusion. Challenge conducted on day 21 was via a 24 h occluded patch. Reactions were read at 24 and 48 h after removing the patch. No sensitisation reactions were observed (Klecak et al., 1977).

6. Another guinea pig maximisation test was conducted using 10 female Hartley albino guinea pigs. Induction consisted of intradermal injection of 10% benzyl salicylate in liquid paraffin and a 48 h occlusive patch with 30% benzyl salicylate in ethanol. The animals were challenged twice with benzyl salicylate at 0.003, 0.01, and 0.03% in ethanol. The second challenge was conducted three weeks after the first challenge. Reactions were read at 24, 48, and 72 h. After the first challenge, no reactions were observed at 24 h, but positive reactions were observed at 48 and 72 h at all doses. Following the second challenge, positive reactions were observed with 0.03% at 24 h and all concentrations at 48 and 72 h (Kashima et al., 1993).

7. A guinea pig open epicutaneous test (OET) was conducted in groups of 6–8 male and female guinea pigs weighing 300–450g. Daily applications of 0.1 mL benzyl salicylate (undiluted or progressively diluted solutions) were made for 3 weeks to a clipped 8.0 $cm^2$ area on the flank of each guinea pig. The test sites were not covered and the reactions were read 24 h after each application. A total of 21 applications of benzyl salicylate in an unspecified vehicle were made for 21 days. Ten control animals were either left untreated or treated with 0.1 mL of the vehicle for 21 days. At the challenge phase, both the test and control animals were treated on days 21 and 35 on the contralateral flank with 30% benzyl salicylate. No sensitisation was produced (Klecak, 1985).

8. An OET was conducted in guinea pigs. Induction consisted of 21 daily open applications to the shaved flank of 6–8 guinea pigs per group. Open challenge applications were made on days 21 and 35. Reactions were read at 24, 48, and 72 h. No reactions were observed with 10% benzyl salicylate (vehicle not provided; no further details provided) (Klecak, 1979).

9. Benzyl salicylate was tested for sensitisation in an OET test in male and female outbred Himalayan guinea pigs (6/8-group) weighing 400–500 g. Animals received 21 daily open applications of 0.1 mL of undiluted and progressively diluted solutions of benzyl salicylate which were applied to an 8.0 $cm^2$ area on the clipped flank. Guinea pigs were challenged by an open application of 0.025 mL benzyl salicylate, in an unspecified vehicle, which was applied to a skin area measuring 2 $cm^2$ on the contralateral flank on days 21 and 35. Reactions were read 24, 48, and 72 h after application. A concentration of 0.03% was reported to be the minimum eliciting concentration and 30% was reported to be the minimum sensitising concentration (Klecak et al., 1977).

10. A closed epicutaneous test (CET) was conducted using 20 guinea pigs. During the induction phase, benzyl salicylate at 30% (vehicle not provided) was applied under occlusion for 48 h on the shaved nape. The same procedure was repeated three times per week for two weeks. Following a 2-week rest period, the animals were challenge with 1% benzyl salicylate under occlusion for 48 h. Three (3/20) sensitisation reactions were observed (Ishihara et al., 1986).

11. The cumulative contact enhancement test (CCET) was conducted using groups of 10 female Hartley albino guinea pigs weighing approximately 250 g. During the induction phase, a 24 h occlusive patch containing 30% benzyl salicylate in ethanol was applied to each animal. The animals were challenged twice ($2^{nd}$ challenge was conducted 3 weeks after the $1^{st}$ challenge) with 1%, 3% and 10% benzyl salicylate in ethanol. Sites were scored at 24, 48, and 72 h. At 1%, one positive (1/10) reaction was observed at 24 h, no

reactions were observed at 48 or 72 h. At 3%, positive reactions (3/10) and (2/10) were observed at 24 and 48 h, respectively. At 10%, positive reactions were observed at 24, 48, and 72 h (Kashima et al., 1993).

12. Tsuchiya et al. (1982) conducted another CCET test on 6–10/group Pirbright and Hartley albino guinea pigs. A 0.2 mL aliquot of 3, 10, 30 (vehicle not reported), or 100% benzyl salicylate was applied to a lint patch which was then applied to the shaved back for 24 h under occlusion. The applications were repeated every other day over a period of 2 weeks. Eleven days after the final induction patch, a challenge was performed. A 0.01 mL aliquot of 50% benzyl salicylate was applied on a circular 2.0 cm in diameter cotton patch to a shaved part of each animal. Reactions were evaluated 24, 48, and 72 h. No sensitisation was observed with induction concentrations of 10 or 100% in Pirbright guinea pigs. Three (3/6) sensitisation reactions were observed when the animals were induced with 30% benzyl salicylate and 1/10 reactions were observed when the animals were induced with 100% benzyl salicylate (Tsuchiya et al., 1982).

13. The cumulative contact enhancement test (CCET) was conducted using 30 tortoise shell guinea pigs weighing 250–300g. Animals were shaved and a 24 h occluded patch with neat benzyl salicylate was applied. Patches were applied every third day for 2 weeks (maximum, 4 applications). An injection of FCA was intradermally administered before the third patch. An untreated group of five animals was used as a control. After an 11 day rest period, a 0.01 mL aliquot of 50% benzyl salicylate in ethanol was applied to a previously untreated site, once daily for 1–3 days. Reactions were evaluated over a period of 43 days. Sensitisation reactions were observed in 13/30 animals (Imokawa and Kawai, 1987).

14. A delayed contact hypersensitivity assay was conducted in 10 female Hartley strain guinea pigs using the AP2 test method. Two induction applications were made 4 days apart which consisted of an intradermal injection with FCA and a 24 h occluded patch at the injection site with 30% benzyl salicylate in ethanol. Two open challenge applications were made on days 11 and 32 with 1, 3, and 10% benzyl salicylate in ethanol. A third challenge application was made with 0.003, 0.01, and 0.03% benzyl salicylate in ethanol on day 39 using a 24 h occluded patch. The reactions were evaluated at 24, 48, and 72 h after challenge. Sensitisation was observed at all three challenges (Kashima et al., 1993).

15. Sensitisation was evaluated in groups of 10 Pirbright guinea pigs weighing 280–350 g using a modified FCA method. Six intradermal injections (2 per day on 3 separate days) of 10% benzyl salicylate in FCA were made into the clipped, shaved shoulder area on days 1, 5, and 9 for a total of 4.5 mg of benzyl salicylate. Challenge was conducted 11 days after induction by applying 0.05 mL of 10% benzyl salicylate in acetone onto the clipped, shaved right flank. Reactions were read at 24, 48, and 72 h. Benzyl salicylate at 10% was a moderate sensitiser (Hausen and Wollenweber, 1988).

16. Sensitisation was evaluated as part of a photo-allergy study using 25 adult albino Dunkin-Hartley guinea pigs. Twenty-four hours prior to application, all animals were clipped free of hair on the back and flanks. On day 1, a topical application of 0.5 mL of benzyl salicylate in absolute ethanol was applied to the middle of the anterior part of the back of the animals for 90 min. On day four of the study, four intradermal injections of 0.1 mL each of FCA diluted at 50% in isotonic saline were made on both sides of the occlusive patch. A second topical application of 0.5 mL benzyl salicylate in absolute ethanol was applied under an occlusive patch for 90 min. A 3$^{rd}$ and 4$^{th}$ topical application under an occlusive patch was made on days 7 and 9 of the study. The challenge was conducted 12 days after the 4$^{th}$ application. A challenge was conducted with 0.5 mL of 10% benzyl salicylate in absolute ethanol applied to a virgin area on the back of the animal for 1.5 h. Reactions were scored at 1, 6, 24, and 48 h. No sensitisation reactions were observed (RIFM, 1983a).

17. The optimisation test was conducted in 20 Pirbright White Strain guinea pigs (10/sex). During the induction period, the animals received one intra-cutaneous injection every other day of 0.1% benzyl salicylate in saline. During the second and third week, benzyl salicylate was incorporated at the same concentration in a mixture of FCA and physiological saline (adjuvant/saline, 1:1 v/v). A total of 10 injections were made. The animals were challenged with 0.1% benzyl salicylate in saline 14 days after the last induction injection using the same procedure. After a further rest period of 10 days, the animals were again challenged but with 10% benzyl salicylate in soft white petrolatum applied under occlusion for 24 h. Reactions sites were scored according to the Draize scale, 24 h after removing the patch. One (1/20) reaction was observed after the intradermal challenge and seven (7/20) reactions were observed after the epidermal challenge (Maurer et al., 1980).

18. The Freund's complete adjuvant test (FCAT) was conducted using outbred Himalayan white-spotted male and female guinea pigs weighing 400–500 g. Induction was via five intradermal injections of 0.1 mL of a 50:50 mixture of benzyl salicylate and FCA into the neck on days 0, 2, 4, 7, and 9. A 24 h closed patch challenge application was conducted on days 21 and 35 at sub-irritant concentration (0.1%). No reactions were observed (Klecak et al., 1977).

19. The sensitisation potential of benzyl salicylate was measured in a guinea pig sensitisation study using a modified Draize procedure. Ten male and female inbred Hartley strain albino guinea pigs/group with an average weight of 350 g were shaved on both flanks. A 0.1 mL aliquot of 1.25% benzyl salicylate, at 2.5 times the ICC (injection challenge concentration: 0.5%), was injected intradermally at four sites which overlap with the 2 axillary and 2 inguinal lymph nodes. The animals were challenged 14 days later by an intradermal injection of 0.1 mL benzyl salicylate into one flank and a topical open application of benzyl salicylate on the other flank at the respective injection challenge concentration of 0.5% and application challenge concentration of 2% (vehicle not provided). Reactions were scored 24 h after challenge treatments. A second challenge was carried out 7 days later. No sensitisation was observed (Sharp, 1978).

20. Benzyl salicylate was tested in a guinea pig sensitisation study using a modified Draize (Draize, 1959) procedure in male and female outbred Himalayan guinea pigs weighing 400–500 g. Induction consisted of ten intradermal injections on alternate days with a dose of 0.05 mL of 0.1% benzyl salicylate in isotonic saline. The animals were challenged on days 35 and 49 with an intradermal injection of 0.05 mL of 0.1% benzyl salicylate in saline. Control animals were also challenged intradermally on days 35 and 49 with benzyl salicylate. Sensitisation was not observed (Klecak et al., 1977).

21. **[Wrong account][4]** A local lymph node assay (LLNA) was conducted in 6–8 week old female CBA/JN mice (4/dose). A 25 µL aliquot of 10% benzyl salicylate in 4:1 acetone/olive oil was applied epidermally to the dorsal portion of the left and right ear lobe of each animal for three consecutive days. A control group was included, which received the vehicle only. On day 6, all mice were injected via the tail vein with 3 H-TdR (20 uCl/250 µL PBS). Five hours after the injection, animals were sacrificed and the draining auricular lymph nodes were excised and pooled for each group. A single cell suspension was prepared and using a quantification of 3 H-TdR incorporation was determined using a beta-scintillation counter. Stimulation Indices (S.I.) based on 1–4 experiments were calculated. Proliferating lymph node cell subpopulations were examined by flow cytometric analysis. The EC3 value was calculated to be 1.5% (375 µg/cm$^2$) (Yoshida et al., 2000).

22. A local lymph node assay was conducted on groups of four female mice. Approximately 25 µL of a 2.5, 5, 10, 25, and 50% w/v preparation of benzyl salicylate in 1:3 EtOH:DEP was applied to the dorsal surface of each ear. The procedure was repeated daily for three consecutive days. Three days after the third application, all the animals were injected via the tail vein with 250 µl of phosphate buffered saline (PBS) containing 20 µlCi of 2.0 Ci/mmol specific activity 3H-methyl thymidine. Approximately 5 h later the animals were sacrificed. The draining auricular lymph nodes were removed from each animal and placed in a container of PBS together with the nodes from the other animals in the group. A single cell suspension was prepared by mechanical disaggregation of lymph nodes through 200-mesh stainless steel gauze. The cell suspensions were then washed three times by centrifugation with approximately 10 mL of PBS. Approximately 3 mL of 5% w/v trichloroacetic acid (TCA) were added and, after overnight precipitation at 4 °C the samples were pelleted by centrifugation and the supernatant was discarded. The cells were then re-suspended in approximately 1 mL of TCA. The lymph node suspensions were transferred to scintillation vials and 10 mL scintillant (Optiphase) was added prior to beta-scintillation counting using a Packard Tri-Carb Liquid Scintillation Counter. Under the conditions of the test, benzyl salicylate was a skin sensitiser. **The EC3 value was calculated to be 2.9%** , corresponding to an area dose of 725 µg/cm$^2$ (RIFM, 2005).

[4] Note by the DS: In the original reference (Yoshida, Y., Oyake, Y., Sakaguchi, H., Okuda, M., Suzuki, H., 2000. Comparison of the effect of allergen and irritant treatment on proliferation and subpopulation of the draining lymph node cells in mice and guinea pigs. The Toxicologist 54, 153, which is an SOT conference abstract), neither benzyl salicylate, nor the numbers reported by (Belsito et al., 2007) and (Lapczynski et al., 2007) are mentioned. Furthermore this abstract deals with the development of a guinea-pig LLNA. It is concluded that the results of this LLNA test (which would point at a classification as Skin Sens. 1A) are highly questionable and therefore they have not been used for this dossier.

### 3.7.2 Human data

#### 3.7.2.1 Comparison mouse LLNA vs. human HRIPT study

**Study reference:**

Api A.M., Basketter D., and Lalko J. (2015): Correlation between experimental human and murine skin sensitization induction thresholds. Cutaneous and Ocular Toxicology 34 (4), 298-302. DOI: 10.3109/15569527.2014.979425

**Detailed study summary and results:**

The LLNA part of this study refers to the results summarised above (Central Toxicology Laboratory, 2005).

Human repeated insult patch tests (HRIPTs) were carried out according to the basic principles; each HRIPT study received approval from an independent ethical review committee (IRB – institutional review board), volunteers were recruited to ensure that a minimum of 100 would complete the study. During the induction phase an occlusive webril/adhesive patch (25 mm Hill Top Chamber System) was used; for benzyl salicylate 0.3 mL of the test material in 1:3 ethanol:diethyl phthalate (vehicle) were applied to each patch. The test material was allowed to volatilise for at least 15 min but no longer than 40 min prior to application to the skin. The left side of the back was used for the test area during the induction phase. Patches remained in place and were kept dry for approximately 24 h, after which time they were removed. A 24 h period, during which no test materials were applied, followed the removal of the Monday and Wednesday patch applications; a 48 h period followed the Friday patch applications. On Mondays, Wednesdays, and Fridays, the test sites were observed and any reactions scored and recorded. The identical test site was then retreated until nine induction applications were completed over a period of approximately 3 weeks. A rest period of approximately 2 weeks followed the last induction patch. No test materials were applied during the rest period. At the challenge phase, the original induction test sites were observed and each subject queried as to whether any reaction had been experienced during the rest period. The untreated right side of the back was used for the for the challenge phase. Patches were applied as in the induction phase and held in place for 24 h after which time they were removed and the challenge site scored. The original test sites were also observed. Scoring of the test sites was also carried out at 48, 72, and 96 h post-patching using the scoring scale: 0 = no visible reaction; +/- = faint, minimal erythema; 1 = erythema; 2 = intense erythema, induration; 3 = intense erythema, induration, vesicles; 4 = severe reaction with erythema, induration, vesicles, pustules. The vehicle was tested separately in the same manner as the test material. The authors calculated the NOEL of this study, which reflects that the outcome yielded no evidence of skin sensitisation. No attempt was made to test at increasing concentrations until skin sensitisation appeared (Api et al., 2015).

#### 3.7.2.2 Review of sensitisation to fragrances

**Study reference:**

Schnuch A., Uter W., Geier J., Lessmann H., and Frosch P.J. (2007): Sensitization to 26 fragrances to be labelled according to current European regulation. Results of the IVDK and review of the literature. Contact Dermatitis 57 (1), 1-10. DOI: 10.1111/j.1600-0536.2007.01088.x

**Detailed study summary and results:**

The multi-centre project IVDK (Information Network of Departments of Dermatology) is an instrument of epidemiological surveillance of contact allergy (CA) in Germany. Patch tests were performed in accordance with the recommendations of the International Contact Dermatitis Research Group and the German Contact Dermatitis Research Group (DKG). Patch test material was obtained from Hermal/Trolab, Reinbek, Germany. Patch test preparations were applied for 24 or 48 h. Readings were done until at least 72 h (grading based on international standards, further refined by the German Contact Dermatitis Group: neg, ?, +, ++, +++, irritant, follicular). The patch test results of every reading, a standardised history (including age, sex, atopic diseases, current and former occupation(s), presumptive causal exposures), along with final diagnoses and site(s) of dermatitis were assessed and documented. Frequencies of sensitisation (as % of patients tested) were calculated both as crude proportions and proportions standardised for sex and age. Subgroups of

patients defined by sensitisation to an index allergen were analysed for concomitant reactions (crude proportions). The reaction index (RI), relating the number of allergic reactions to the number of doubtful or irritant reactions, ranging from RI = -1 (all reactions non-allergic) to RI = +1 (all reactions being allergic), and the positivity ratio (PR), as the proportion (%) of "+" reactions out of the total number of allergic reactions, were calculated as parameters to assess the patch test preparation. A low RI (e.g. 0.8) together with a high PR (e.g. 100%) is indicative of a 'problematic' patch test preparation where a number of the '+' reactions may be suspected to be falsely positive. For data management and analysis, the statistical software package SAS (version 9.1, SAS Institute, Cary, NC, USA) was used. Results of patch testing for benzyl salicylate 1% frequencies of sensitisation = 2 / 2041 (0.1 % of positive reactions). The calculated frequency of allergic reactions, standardised for age and sex (% Pos. std) was also 0.1% while 95% confidence interval (95% CI) was 95% CI = 0.0–0.2. The reaction pattern of the patch test preparations (irr: irritant; f: follicular; ?: doubtful; RI: reaction index; PR: positivity ratio): irr./? = 7; f= 1; + = 2; ++ = 0 ; +++ = 0 ; RI (reaction index) = 0.6 ; PR (positivity ratio) = 100.0. The authors classified benzyl salicylate as a "very rare allergen" (Schnuch et al., 2007).

### 3.7.2.3 Reviews[5] citing[6] further animal data

**Study references:**

Belsito D., Bickers D., Bruze M., Calow P., Greim H., Hanifin J.M., Rogers A.E., Saurat J.H., Sipes I.G., and Tagami H. (2007): A toxicologic and dermatologic assessment of salicylates when used as fragrance ingredients. Food and Chemical Toxicology 45 (1 SUPPL.), S318-S361. DOI: 10.1016/j.fct.2007.09.066;

Lapczynski A., McGinty D., Jones L., Bhatia S., Letizia C.S., and Api A.M. (2007): Fragrance material review on benzyl salicylate. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 45 Suppl 1, S362-380. DOI: 10.1016/j.fct.2007.09.036

**Detailed study summary and results:**

#### 3.7.2.3.1 Maximisation and repeated insult tests in volunteers

For a tabular overview, cf. Table 11 of the main dossier. Full references have not been included in the reference list of this dossier, but can be accessed via the reference lists in the above two reviews. Study numbers below refer to the numbers given in (Belsito et al., 2007; Lapczynski et al., 2007).

1. A Human Repeated Insult Patch Test (HRIPT) was conducted in 101 volunteers (29 males and 72 females). During the induction phase, 0.3 mL of benzyl salicylate were applied to a webril/adhesive patch (25 mm Hilltop Chamber System), and then applied to the back of each volunteer. Patches remained in place for approximately 24 h. Nine induction patches were completed over a 3-week period. After a 2-week rest period, challenge patches were applied to a virgin site on the back and kept in place for 24 h. The test sites were scored at 48, 72, and 96 h. Under the conditions of the study, 15% benzyl salicylate in 3:1 DEP:EtOH did not induce dermal sensitisation (RIFM, 2004a).

2. To evaluate the potential for cross-reactivity an HRIPT was conducted on 103 volunteers (29 male and 74 females). Using the same method as described above, subjects were induced with 30% hexyl salicylate in 3:1 DEP:EtOH, and cross-challenged with 15% benzyl salicylate in 3:1 DEP:EtOH. No cross-reactions were observed (RIFM, 2004b).

3. Thirty-five subjects (17 males and 18 females) completed a HRIPT with 10% benzyl salicylate in alcohol SDA 39C. An aliquot of 0.5 mL benzyl salicylate was applied to a 1 x 1 inch Webril swatch affixed to the center of a 1 x 2 inch elastic bandage. These patches were then applied to the upper arms for 24 h under semi-occlusion. 9 induction applications were made over a 3-week period. After a 2-week rest , a 24 h challenge application was made to the same site and to a virgin site in the same way as for induction. Reactions were scored 48 and 96 h post-application. No sensitisation was observed (RIFM, 1975c).

[5] Since the two reviews mostly address the same studies, they are reported jointly.

[6] With the exception of the studies mentioned elsewhere in the main dossier or this annex, the references cited in these reviews have not been accessed directly by the DS.

4. An HRIPT was conducted on 52 volunteers using a modified Draize method. An aliquot of 5 mL of 5% benzyl salicylate in dimethyl phthalate was applied to a patch which was then applied to the inner surface of the right deltoid area of each subject and secured by means of overlying strips of impervious adhesive tape, which were then further occluded with additional overlying strips of similar tape. The patches remained in place for 48 h, when they were removed, observed, and recorded. A series of ten induction patches were applied. The challenge patches were applied after a 2-week rest period in the same manner as the induction patches except they were applied in duplicate, one set to the inner surface of each deltoid area. Patches remained in place for 48 h. Reactions were read at patch removal and again at 72 and 144 h. No sensitisation reactions were observed (RIFM, 1968).

5. A human maximisation test was carried out with 20% benzyl salicylate in petrolatum on 25 male and female volunteers. Application was under occlusion to the same site on the volar forearms of all subjects for five alternate day, 48 h periods. After a 14-day rest period, a challenge patch was applied. Reactions were read at 48 and 96 h. Sensitisation was observed in 2/25 volunteers (RIFM, 1980).

6. A human maximisation test was carried out in 25 healthy male and female volunteers with 30% benzyl salicylate in petrolatum. Benzyl salicylate was applied under occlusion to the same site on the volar forearms of each subject for five alternate day, 48 h periods. Patch sites were pre-treated for 24 h with 5% aqueous sodium lauryl sulfate (SLS) under occlusion. After a rest period, a challenge patch was applied. Challenge sites were read at patch removal and 24 h later. No sensitisation reactions were observed (RIFM, 1975a).

7. A maximisation test was conducted with 30% benzyl salicylate in petrolatum. Benzyl salicylate was applied to the same site on the volar forearm of 25 male volunteers under occlusion for five alternate day, 48-h periods. Each application was preceded by 24 h occlusive applications of 5% aqueous SLS. Following a 10-day rest period, challenge patches of benzyl salicylate were applied to fresh sites on the backs of each subject under occlusion for 48 h. The challenge sites were pre-treated for 1 h with 10% aqueous SLS. Challenge sites were read at 48 and 72 h. No sensitisation reactions were observed (RIFM, 1970c).

8. A maximisation test was conducted on 25 healthy Japanese-American volunteers using 20% benzyl salicylate in petrolatum which was applied under occlusion to the volar forearms of all subjects for five alternate day, 48 h periods. The patch site was pre-treated for 24 h with 5% aqueous SLS under occlusion. Following a 10–14 day rest period, a challenge patch of benzyl salicylate was applied to fresh sites for 48 h under occlusion. The challenge sites were pre-treated for 30 min. with 3% aqueous SLS under occlusion on the left side of the back whereas benzyl salicylate was applied without SLS on the right side. Additional SLS controls were placed on the left and petrolatum on the right. One out of 25 volunteers showed a sensitisation reaction (RIFM, 1979).

9. A maximisation test was carried out in 22 healthy male volunteers. Benzyl salicylate at 30% in petrolatum was applied under occlusion to the same site on the forearms of all subjects for five alternate day, 48 h periods. Patch test sites were pre-treated for 24 h with 5% aqueous SLS under occlusion for the initial patch only. Following a 10–14 day rest period, a challenge patch was applied to a fresh site for 48 h under occlusion. The challenge sites were pre-treated for 30 min with 2% aqueous SLS under occlusion on the left side of the back whereas the benzyl salicylate was applied without SLS on the right side. Reactions to challenge were read 48 and 72 h after patch removal. Benzyl salicylate produced no sensitisation reactions (RIFM, 1975b).

#### 3.7.2.3.2 Diagnostic patch tests in patients

For a tabular overview, cf. Table 12 of the main dossier. Full references have not been in cluded in the reference list of this dossier, but can be accessed via the reference lists in the above two reviews. Study numbers below refer to the numbers given in (Belsito et al., 2007; Lapczynski et al., 2007).

1. Closed patch tests were conducted in 313 patients with 0.05–0.5% benzyl salicylate in a base cream or in 99% ethanol. Patches consisted of a piece of 1.0 $cm^2$ lint with a 2.0 $cm^2$ cellophane disc placed on the lint and covered with a 4.0 $cm^2$ plaster. Patches were applied to the back, the forearm, and the inside of the upper arm for 24 to 48 h. Reactions were read 30 min after patch removal. Erythema was observed in 5 out of 313 (1.3%) patients (Takenaka et al., 1986).

2. Patch testing was conducted using 394 subjects with contact dermatitis, cosmetic dermatitis, eczema, seborrheic dermatitis, and facial dermatitis. Test patches with 1, 2, or 5% benzyl salicylate in petrolatum were applied to the back of each subject with Finn Chambers and Scanpor tape. Test sites were read on days 2 (day after application), 3, and 7, and scored according to JCDRG and ICDRG standards. Questionable reactions were observed at all concentrations, and a positive reaction (++) was observed at 5% (no further details provided) (Ueda, 1979; Ueda, 1994).

3. The principle patch test results of the North American Contact Dermatitis Group for the period from July 1, 1975 to June 30, 1976 have been reported. A total of 183 patients were patch tested with fragrance allergens. Test materials were applied with A1 Test strips or Finn Chambers for 48 h in vertical rows affixed with 2-inch wide occlusive tape. Reactions were read at 48 and 96 h. Reactions to 2% benzyl salicylate (vehicle not reported) were observed in 2.1% of the 183 patients tested (Rudner, 1977; Rudner 1978).

4. Ferguson and Sharma (1984) reported the results of patch tests conducted in 241 patients (180 females and 61 males) from October 1981 to 1983. Patients were patch-tested for sensitivity to fragrances in a perfume screening series. The Finn Chamber technique was used. Reactions to 2% benzyl salicylate in paraffin were observed in 6/241 patients and were characterised by erythema and edema.

5. Fifty patients with photosensitivity dermatitis with actinic reticuloid (PD/AR) syndrome, 32 subjects with polymorphous light eruption (PLE) and 457 with contact dermatitis (CD), were studied to determine the incidence of contact allergic sensitivity to some common fragrance materials. Each subject was patch-tested to various fragrance materials using a standard closed patch test technique. A total of 10mg of the test material supplied in paraffin was applied to standard Al-Test strips which were then placed on the skin of the upper back, secured with Scanpor adhesive tape, and removed at 48 h. Reactions were read at patch removal and then again at 72 h. Benzyl salicylate at 2% in yellow paraffin produced one reaction in (1/457) CD patients. No reactions were observed in PD/AR or PLE patients (Addo et al., 1982).

6. In a multicenter study conducted from September 1998 to April 1999, 1,825 patients were patch tested with nine fragrance allergens and the fragrance mix. The test procedures and concentrations were carried out according to internationally accepted criteria and published studies. Positive reactions to 2% benzyl salicylate in petrolatum were observed in 10 (0.5%) patients (deGroot et al., 2000).

7. In a multicenter study conducted in North America from January 1980 to May 1987, 19 patients with eyelid dermatitis and 70 patients with dermatitis at other sites were patch tested with 2% benzyl salicylate in petrolatum. Benzyl salicylate was applied to Al-Test strips or Finn Chambers, which were applied to the upper back and secured to the skin with Scanpor for a period of 48–72 h. Reactions were read at patch removal and re-examined in the majority of cases between 48 and 96h after patch removal. Sites were scored according to the ICDRG scoring system. Positive reactions were observed in 5.3% of the 19 eyelid dermatitis patients. No other reactions were observed (Nethercott et al., 1989).

8. From January to August 1982, 31 fragrance materials were patch-tested in order to determine their incidence of positive reactions. Benzyl salicylate at 2% (vehicle not provided) produced positive reactions in 13/200 subjects (Asoh et al., 1985a).

9. Hayakawa (1986) reported the incidence of positive patch tests conducted in 1984 by the Japan Patch Test Research Group. Forty-eight hour closed patch tests with cosmetic ingredients were conducted on patients with cosmetic dermatitis. Reactions were read 24 h after patch removal. Reactions to 2% benzyl salicylate in petrolatum were observed in 5/157 patients.

10. In patch tests conducted from 1981 to 1983, the incidence of positive reactions to 2% benzyl salicylate in petrolatum causing allergic contact dermatitis of the delayed type, based on the European and North American Standard Test series for patch testing, was 38/788, or 4.8% (Sugai, 1986).

11. In a series of patch tests conducted from 1978 to 1986 with cosmetic ingredients in patients with eczema or dermatitis, 5% benzyl salicylate (vehicle not reported) produced reactions in 4.0% (30/756) patients (Itoh et al., 1988).

12. A total of 155 patients with cosmetic dermatitis and female facial melanosis were patch tested with various fragrance materials. The test samples were applied on the cloth disks of Torii's adhesive plaster and the plaster was applied to the upper back of the patient for 48 h. Reactions were assessed at 1 h, 24 h, 1 week

and 2 weeks after removal. Positive responses to 5% benzyl salicylate in petrolatum were observed in 12 out of 155 patients (Itoh, 1982).

13. **[Unreliable account]**[7] A 1979 survey conducted by the SDA (The Soap and Detergent Association) produced 10,538 patch test and repeated patch test results on 8430 subjects. Benzyl salicylate was tested in consumer products (maximum concentration used reported 0.2%), and in fragrance blends (maximum concentration reported was 1%), and was also tested alone (10%) in ethanol. No reactions were observed (Kohrman et al., 1983).

14. Benzyl salicylate was patch-tested in dermatitis patients at 1, 2, and 5% in petrolatum. At 1%, reactions were observed in 4/51 (7.8%) melanosis patients and 1/129 (0.8%) cosmetic dermatitis patients. At 2%, reactions were observed in 7/51 (13.7%) melanosis patients and 2/129 (1.6%) cosmetic dermatitis patients. At 5%, reactions were observed in 10/51 (19.6%) melanosis patients, 5/129 (3.9%) cosmetic dermatitis patients and 1/84 (1.2%) non-cosmetic dermatitis and/or eczema patients (Ishihara et al., 1979).

15. The Mid-Japan Contact Dermatitis Research Group conducted a study to determine the optimal patch testing concentration of benzyl salicylate. In the first series, 394 patients were patch-tested with various fragrance materials including 1, 2, and 5% benzyl salicylate in petrolatum. Six positive (6/394) reactions were observed at 1%, nine (9/394) at 2% and 23 (23/394) at 5%. In the second series, patch tests were conducted using 1%, 2% and 5% benzyl salicylate in petrolatum in 21 subjects using the same procedure as above. No reactions were observed at 1 and 5%, two (2/21) reactions were observed at 2% (Ueda, 1979).

16. Between 1978 and 1985, eczema and dermatitis patients were patch-tested with various synthetic perfumes. A total of 680 patients were patch tested with benzyl salicylate at 5% (vehicle not provided). Positive reactions were observed in 27/680 patients (Itoh et al., 1986).

17. The Mid-Japan Contact Dermatitis Research Group conducted a study to determine the optimal patch-testing concentration of benzyl salicylate. A total of 212 patients were patch tested with various fragrance materials including 5% benzyl salicylate in petrolatum. Positive reactions were observed in 12/212 patients (Hada, 1983).

18. From 1989 to 1992, 332 patients (25 male and 307 female) suspected of cosmetic contact dermatitis were patch tested with various cosmetics and their ingredients. Of these patients, 103 were patch tested with 2% benzyl salicylate (vehicle not provided). Positive reactions were observed in 2/103 patients (Fujimoto et al., 1997).

19. A total of 315 consecutive hand eczema patients were patch-tested with various fragrance materials. No reactions were observed to 5% benzyl salicylate in petrolatum (Heydorn et al., 2002).

20. In 1994, patients with suspected contact dermatitis from cosmetic products were patch tested with cosmetic ingredients. One positive (1/386) reaction was observed to 2% benzyl salicylate in petrolatum (Sugai, 1996).

21. Patients with contact dermatitis were patch-tested with 0.1 (65 patients) and 1% (201 patients) benzyl salicylate in petrolatum. Patch tests were conducted using Finn Chambers and Scanpor tape. Three reactions (3/201) were observed with 1% and one (1/65) was observed with 0.1% benzyl salicylate (Kozuka et al., 1996).

22. The Japan Contact Dermatitis Research Group conducted a study to determine the optimal patch-testing concentration of benzyl salicylate. A total of 357 patients at 16 different centers were patch-tested with various fragrance materials that included 5% benzyl salicylate in petrolatum. Patch tests were conducted using Finn Chambers and were secured with Scanpor tape. Reactions were observed in 14/176 patients (Shoji, 1982).

23. Dermatitis Patients (2,272) were tested with the standard series and 445 had a positive reaction to balsam Peru. Out of these (445) patients, 102 were patch tested with the balsam Peru series and propolis. Patch tests were applied to the backs for 24 h using Finn Chamber and Scanpor tape. Reactions were read after 24 and

[7] The DS notes that in Kohrman (Kohrman et al., 1983), no patch test experiments with benzyl salicylate alone seem to have been reported.

72 h according to ICDRG rules. Reactions to 2% benzyl salicylate in petrolatum were observed in 3/102 patients (Hausen, 2001).

24. A total of 747 patients suspected of fragrance allergy were patch tested with a special fragrance series which was comprised of the eight constituents from the fragrance mix. Reactions were assessed at 72 h and scored according to criteria established by ICDRG. Three positive reactions (3/747) were observed with benzyl salicylate at 1% in petrolatum (Wohrl et al., 2001).

25. From January 1992 to June 1993, the incidence of positive reactions to various fragrance materials was investigated in patients with contact dermatitis. Patients were patch tested with 10 fragrance materials. Reactions were assessed according to rules established by ICDRG. Positive reactions were observed in 7/706 subjects with 2% benzyl salicylate in petrolatum (Katoh et al., 1995).

26. A total of 658 patients with hand eczema, who had reacted positively to fragrance materials in the European Standard series, were further patch-tested with a selection of fragrances. A positive reaction was observed in 2/658 patients with 5% benzyl salicylate in petrolatum (Heydorn et al., 2003).

27. In a series of patch tests conducted in 1255 patients with contact dermatitis from 1973 to 1981, reactions to 2% benzyl salicylate in petrolatum were observed in 6.1% (77/1,255) patients (Sugai, 1982).

28. A closed patch test was conducted on a group of Japanese male and female subjects, with 10% of the test population being characterised as eczema-prone and allergic persons. A 1.0 $cm^2$ patch was applied to the inside of the upper arm and flexor of the forearm then affixed with adhesive tape. Patches were applied for 24 h. The vehicle was a perfume-based cream. Reactions were read 30 min after removal. Positive reactions to 0.2% benzyl salicylate were observed in 3/313 patients and questionable reactions were observed in 2/313 patients tested (RIFM, 1974).

29. A total of 212 patients with cosmetic dermatitis, 35 patients with facial melanosis, and 275 patients with non-cosmetic dermatitis or eczema were patch tested with 5% benzyl salicylate (vehicle not reported). In addition, 101 subjects used as controls were also tested with 5% benzyl salicylate. Reactions to 5% benzyl salicylate were observed in 8/212 cosmetic dermatitis patients, 7/35 facial melanosis patients, 9/275 non-cosmetic dermatitis and eczema patients and 1/101 controls (Nishimura et al., 1984).

30. From December 1981 to November 1982, 181 cases of dermatitis patients with melanosis faciei feminae were patch tested with their cosmetic products and 137 allergens. Positive reactions to 5% benzyl salicylate in petrolatum were observed in 25/181 patients (Hayakawa et al., 1983).

31. A total of 394 subjects, most of whom suffered from various facial dermatoses, were patch-tested for 48 h under occlusion with benzyl salicylate at concentrations of 1, 2, or 5% in petrolatum. Reactions were read 1 h after patch removal and again the next day. Reactions were assessed using ICDRG guidelines. At 1%, irritant reactions were observed in 4.6% of the subjects and allergic reactions were observed in 1.5% of the subjects. At 2%, irritant reactions were observed in 3.3% while allergic reactions were observed in 2.3% of the subjects. At 5%, irritation was observed in 4.8% of the subjects and allergic reactions were observed in 5.8% of the subjects (MJCDRG, 1984).

32. From 1990 to 1991, 64 patients with cosmetic dermatitis, 7 facial melanosis patients and 32 non-cosmetic dermatitis patients were patch-tested with 5% benzyl salicylate in white petrolatum. A positive reaction to 5% benzyl salicylate was observed in 1/64 (1.6%) cosmetic dermatitis patient. No reactions were observed in facial melanosis patients and non-cosmetic dermatitis patients (Haba, 1990).

33. From September 1992 to August 1993, a series of patch tests to most allergenic ingredients of cosmetic and toiletry products were conducted. A total of 482 patients were tested with 2% benzyl salicylate in petrolatum. Positive reactions were observed in 4/482 patients (Nagareda et al., 1996).

34. Nagareda et al. (1992) reported the incidence of positive reactions to 17 ingredients derived from patch tests conducted during 1990 to 1991, in patients with contact dermatitis. Patch tests were conducted using Finn Chambers and Scanpore tape. Positive reactions were observed with 2% benzyl salicylate in petrolatum in 8/436 contact dermatitis patients.

35. Patch tests conducted on patients from 1971 to 1980 using 5% benzyl salicylate in petrolatum resulted in positive reactions in 11% of cosmetic dermatitis and 1% of non-cosmetic dermatitis patients from 1971 to

1974, in 25% cosmetic and 0% non-cosmetic dermatitis patients from 1975 to 1977, and in 11% cosmetic and 0% non-cosmetic dermatitis patients from 1978 to 1980 (Nakayama et al., 1984).

36. In a world-wide multicenter study to investigate fragrance sensitisation in patients with suspected fragrance allergies, 167 patients were patch-tested with benzyl salicylate which was applied to the upper back with Finn Chambers and Scanpor for a period of 48–72 h. Reactions were read at patch removal and then re-examined between 48 and 120 h after patch removal. Reactions were scored according to the North American Contact Dermatitis Group's modification of the ICDRG scoring criteria. Benzyl salicylate, at 2% in petrolatum, produced irritant reactions in 3% of the patients and allergic reactions in 3% of the patients. At 5%, irritant reactions were observed in 3% of the patients and allergic reactions were observed in 4.8% of the patients (Larsen et al., 1996a).

37. Frosch et al. (1995b) reported the results of a multi-center study on patch tests with 48 fragrance materials. Benzyl salicylate was applied to the back with Finn Chambers and Scanpor for 2 days. Reactions were assessed as per ICDRG guidelines on days 2 and 3 or days 2 and 4. Benzyl salicylate at 1 and 5% in petrolatum was tested in 100 patients (64 females and 36 males). No reactions were observed with 1%. One (1/100) questionable reaction was observed with 5%.

38. Patch tests were conducted on patients with and without cosmetic dermatitis as well as patients with facial melanosis, from the period 1978 to 1980. The vehicle was not reported. Positive reactions were observed with 5% benzyl salicylate in 7.7 (12/155), 4.4 (7/159), and 2.1% (1/48) of subjects with prior histories of cosmetic dermatitis, eczema and dermatitis, or no prior condition, respectively (Ishihara et al., 1981).

## 3.7.3 Other data

### 3.7.3.1 In vitro method to estimate the sensitisation induction level

**Study reference:**

Galbiati V., Papale A., Marinovich M., Gibbs S., Roggen E., and Corsini E. (2017): Development of an *in vitro* method to estimate the sensitization induction level of contact allergens. Toxicology Letters 271, 1-11. DOI: 10.1016/j.toxlet.2017.01.016

**Detailed study summary and results:**

All reagents and chemicals were purchased from Sigma–Aldrich Co. (St. Louis, Mo, USA) at the highest purity available. Cell culture medium (EPI-100-MM-60) was supplied by MatTek Corporation. For the reconstituted human epidermis (RhE), the EpiDerm™ (MatTek In Vitro Life Science Laboratories, Bratislava, Slovak Republic) was used produced from normal human keratinocytes (neonatal male skin donors) and maintained according to the suppliers' instructions at 37 °C, 5% $CO_2$ and 95% relative humidity. After 24 h of equilibration time, RhE were exposed to chemicals in order to determine the $EC_{50}$ value of the chemical ($EC_{50}$ = effective chemical concentration required to reduce RhE metabolic activity to 50% of the maximum value compared to vehicle-exposed cultures) and the release of IL-18 associated with its treatment. The vehicle with the highest dissolving capacity was chosen; chemicals were then tested in dose-response experiments starting with the highest soluble concentration and decreasing with 2-fold serial dilutions for a total of five concentrations. Test chemical stock solutions were freshly prepared in acetone:olive oil (4:1) or in medium.

For exposure, Finn Chamber filter paper discs 8 mm (Epitest LTD Oy, Finland) were impregnated with chemicals or vehicle controls and applied topically to the RhE stratum corneum. Cultures were incubated with chemicals for 24 h. After chemical exposure, filter paper discs were removed and metabolic activity was determined immediately by the MTT assay, while culture supernatants were harvested and stored at −80 °C for IL-18 quantification by ELISA. Chemicals were tested in single in two independent RhE batches. Control conditions (unexposed, vehicle(s) and positive control (DNCB 3 mg/mL in AOO)) were tested in double in each experiment. After 24 h exposure, filter paper discs were removed from RhE. The MTT analysis was performed in 24-well plates. Thiazolyl blue tetrazolium bromide (MTT) (Sigma) solution in

PBS (0.5 mL/well of a 5 mg/mL solution) was added to the 24-well plate, RhE cultures were placed on top and further incubated for 2 h under standard culture conditions. Cultures were then transferred to a new 24-well plate containing 0.3 mL isopropanol to dissolve the formazan crystals. Plates were incubated overnight, sealed with parafilm and protected from the light at room temperature. The absorbance was measured at 595 nm and cell viability was expressed as % relative to the absorbance value of vehicle control-treated RhE. The RhE-$EC_{50}$ values were obtained by linear regression analysis based on changes in metabolic activity (MTT).

ELISA plates were coated with capture antibody (Mouse anti-human IL-18-UNLB, Southern Biotech, AL, USA) 0.1 µg/mL in PBS (dilution 1:5000–100 µl/well) and incubated at 4 °C. After blocking for 1 h in 1% BSA in PBS, samples and standard curve (recombinant human IL-18, MBL International, MA, USA) were plated (100 µl/well) for 2 h at RT. After washing, rabbit anti IL-18 (MyBioSource, CA, USA) was added (dilution 1:1000 in blocking buffer – 100 µl/well) for 1 h at RT. After 1 h, a goat anti-rabbit IgG-horseradish peroxidase conjugate (BIO-RAD, Segrate, Italy) was added (dilution 1:15.000 in blocking buffer – 100 µl/well). After washing, the substrate solution (tetramethylbenzidine liquid substrate, Sigma–Aldrich, MO, USA) was added (100 µl/well). Plates were read at 595 nm.

Results were calculated in pg/mL from a standard curve and then converted to a stimulation index (SI), calculated as: IL18SI = (IL18 pg/mL in chemical-treated RhE)/(IL18 pg/mL in vehicle-treated RhE). IL-18 SI2 values were obtained by linear regression analysis based on the chemical concentration resulting in a 2-fold increase in IL-18 release. Testing was performed in at least two independent experiments using different RhE batches; the results are incorporated into a prediction model. The following readouts were used: For the assessment of allergenicity (YES/NO): if the fold increase in intracellular IL-18 was ≥ 2.0 in at least one of the concentrations tested, the chemical was classified as a contact sensitiser (H317). In case the two experiments gave inconsistent results (e.g. one positive and one negative), a third experiment was done, and the majority was used for classification (e.g. if two positives and one negative, the classification will be contact allergen). For potency assessment, the arithmetic means of the values obtained in the two experiments for both cytotoxicity (MTT assay) expressed as of $EC_{50}$ and IL-18 SI2 (chemical concentration resulting in 2 fold increases in IL-18 release) were considered. Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

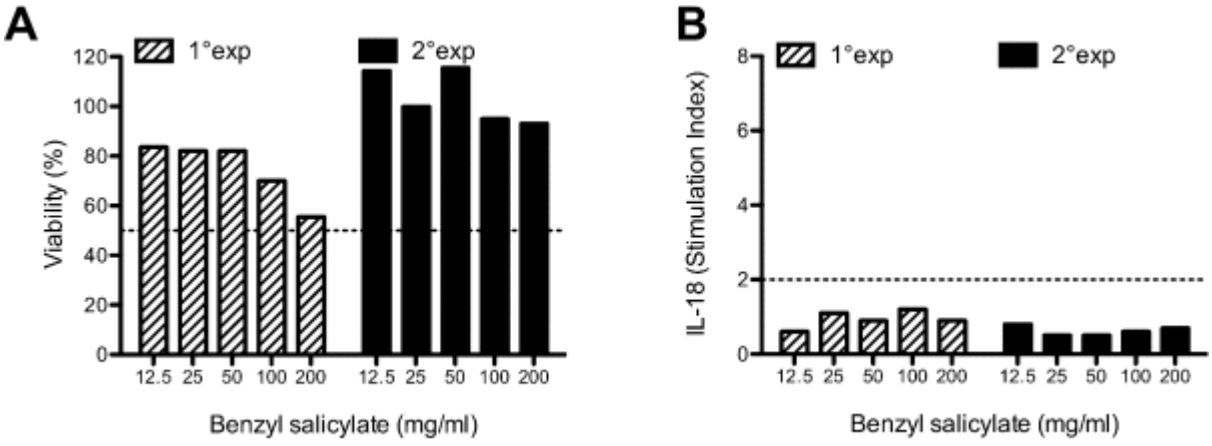


Figure 1: Effects on cell viability and IL-18 release of benzyl salicylate. RhE was treated for 24 h with increasing concentrations benzyl salicylate (A, B),Viability was assessed by MTT while IL-18 by ELISA. Viability (A, C, E) is expressed as % vs vehicle treated RhE (100%), and IL-18 as stimulation index (SI) compared to vehicle treated RhE (B, D, F). The dotted lines are set at 50% viability and IL-18 SI-2. From (Galbiati et al., 2017).

The data presented by the authors on benzyl salicylate are confusing and cannot be used "per se" to evaluate the sensitising potential. Main weaknesses of the study: Testing was done in single in only two independent RhE batches, exposure was only for 24 h, no standard deviation was provided, no statistics and no validation of the experiments are reported, there was a high variability between experiments, background cytotoxicity in the two experiments was quite different, and the effects of the test substance or cytotoxicity of the vehicle were not shown. Furthermore, a modified ELISA

was used for the evaluation test that was not validated before, while the correlation between cytotoxicity and the strength of IL-18 release is not always straightforward. This model might predict extreme and strong sensitisation and thus is not appropriate for the case of benzyl salicylate. Cf. also Figure 1 (Galbiati et al., 2017).

#### 3.7.3.2 EpiSensA asssay for the assessment of the regulation mechanism of marker genes in NHEKs

**Study reference:**

Saito K., Takenouchi O., Nukada Y., Miyazawa M., and Sakaguchi H. (2017): An in vitro skin sensitization assay termed EpiSensA for broad sets of chemicals including lipophilic chemicals and pre/pro-haptens. Toxicology in Vitro 40, 11-25. DOI: 10.1016/j.tiv.2016.12.005

**Detailed study summary and results:**

Benzyl salicylate was purchased from Sigma–Aldrich and dissolved in AOO (acetone (Sigma-Aldrich):olive oil (Kanto Chemical) = 4:1). NHEKs (KURABO, Osaka, Japan) were cultured in HuMedia–KG2 (KURABO), supplemented with insulin, bovine pituitary extract, epidermal growth factor, hydrocortisone, kanamycin, and amphotericin B. When used for experiments, cells were seeded in 12-well plates at a density of $1.0 \times 10^5$ cells per well in 1 mL of culture medium, distilled water (DW; Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan), or 50% ethanol in DW (50% EtOH; ethanol was purchased from Kanto Chemical) when applied to the RhE model, since all of these vehicles have often been adopted in dermal application in animal testings and failed to affect cytotoxicity under the testing conditions used in this study. The CAS No., LLNA Estimated Concentration of a test substance needed to produce a stimulation index of three (EC3) values (%) (OECD, 2010), logKow calculated by KOWWIN ver.1.68 in EPI suite™ (Environmental Protection Agency, Washington, DC, USA), and vehicle for each chemical are listed The RhE model "LabCyte EPI-MODEL (24well format)" (Japan tissue Engineering Co. Ltd., Aichi, Japan) was used that consists of NHEKs (neonatal foreskins) which construct a multi-layer structure consisting of a fully differentiated epithelium with features of a normal human epidermis, including a stratum corneum. The tissues were pre-cultured overnight at 37 °C (5% $CO_2$) in 0.5 mL/tissue of culture media provided by the manufacturer.

Following 6 h of treatment with chemicals, the cells were lysed with RLT buffer (Qiagen) containing 2.5% DL-Dithiothreitol (Sigma-Aldrich). The RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and was then quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at −80 °C until use. The Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) was used to prepare cDNA. The mixture consisted of 1 µL of a 10 mM dNTP mix, 1 µL of Oligo (dT) (0.5 µg/µL), RNase free water (the above 3 reagents were supplied by the manufacturer) and 0.5 µg of total RNA (variable volume) was added to achieve a total volume of 10 µL. The mixture was incubated at 65 °C for 5 min and then on ice for 1 min. A mixture of 10× RT Buffer (2 µL), 25 mM $MgCl_2$ (4 µL), 0.1 M DTT (2 µL), and RNase Out (1 µL) was added to the reaction and incubated at 42 °C for 2 min. Subsequently, 1 µL of Super Script III was added and incubated at 50 °C for 50 min. The reaction was terminated by incubation at 85 °C for 5 min. The cDNA was treated with 1 µL RNase H for 20 min at 37 °C and was then stored at −20 °C. The primers and probes for five genes: activating transcription factor 3 (ATF3); DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4); glutamate-cysteine ligase, modifier subunit (GCLM), interleukin-8 (IL-8), and Nrf2, and for one endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) were designed by Assays-by-Design Service from Applied Biosystems and the sequence information remains confidential. The primers and probes were delivered as a 20×Taqman Gene Expression Assay mix (Applied Biosystems). A total volume of 20 µL sample, which consisted of 1 µL TaqMan® Gene Expression Assay 20×, 10 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µL cDNA template and 8 µL $dH_2O$ was prepared and applied to an optical reaction plate (96-well plate; Applied Biosystems). Real-time PCR reactions were performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Relative gene expression levels versus control (fold change) were calculated using the $2^{-\Delta\Delta Ct}$ method. Student's t-test was used to evaluate statistical significance. p- Values < 0.01 were considered to be statistically significant.

Solid test chemicals were dissolved in an appropriate vehicle (AOO, DW, or 50% EtOH) at the maximum soluble concentration (from 50%) and 4-fold serial dilutions were then made to prepare test chemical solutions at doses ranging from the maximum soluble concentration to a minimum concentration of 0.02%. Liquid test chemicals were serially diluted 4-fold with an appropriate vehicle to prepare test chemical solutions at doses ranging from 100% (neat chemical) to a minimum dose of 0.02%. A 5 µL aliquot of each test chemical solution was applied to the surface of the LabCyte EPI-MODEL (1 well per group) and was incubated for 6 h at 37 °C (5% $CO_2$). An untreated and a killed control tissue exposed to 10 µL of 10% triton (1 well per group) were prepared as control tissues for cell viability measurement. Cell viability was assessed using the lactate dehydrogenase assay (LDH assay). For the main study, each test chemical was dissolved at the minimum concentration that showed 80% cell viability in the pre-screening. The test chemical solutions were prepared by making 2-fold serial dilutions of the maximum concentration that showed 90% cell viability in the pre-screening.

Four to five different concentrations were prepared, if no cytotoxicity was observed, at least three test solutions of different concentrations were prepared. A 5 µL aliquot of each test chemical solution was applied to the surface of the LabCyte EPI-MODEL (3 wells per group) and was incubated for 6 h at 37 °C (5% $CO_2$). An untreated tissue, a killed control tissue exposed to 10 µL of 10% triton, and vehicle treated tissues (3 wells per group) were prepared as control tissues. Following 6 h treatment with chemicals, the tissue surfaces were rinsed three times in 500 µL pre-warmed D-PBS(−) (Life Technologies, Carlsbad, CA, USA). The tissues were then gently removed, placed into a 1.5 mL microtube containing 0.5 mL TRIzol® (Invitrogen) and vortexed. Chloroform (100µL; Tokyo Chemical Industry, Tokyo, Japan) was added to each microtube containing a homogenised sample. The samples were centrifuged at 12,000 ×g for 15min at 4 °C. The aqueous phase containing RNA was transferred to a 1.5 mL microtube and RNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified using a ND-1000 spectrophotometer and was stored at −80 °C until use.

To determine cell viability, LDH activity in the culture media was measured using an LDH cytotoxicity detection kit (Takara Bio, Inc., Tokyo, Japan) in accordance with the manufacturer's instructions. Upon LDH release, this kit creates a red formazan dye that absorbs light at 490 nm. The absorbance of each well was measured at 490 nm and 620 nm (the reference wavelength) using a plate reader (BMG LABTECH GmbH, Offenburg, Germany). ΔAbsorbance was calculated by subtracting the absorbance at 620 nm from the absorbance at 490 nm. Media from an untreated tissue or a tissue treated with 10% Triton X-100 was used for calculation of minimal LDH release (negative control) or maximal LDH release (killed control), respectively. Cell viability was calculated using the following formula: Cell viability=100−(ΔAbsorbance of negative control−ΔAbsorbance of a chemical−treated group) /(ΔAbsorbance of killed control−ΔAbsorbance of negative control) X 100. The mean value (3 wells per group) of maximal fold induction ($I_{max}$) of each gene was determined using data obtained from chemical concentrations that displayed over 80% cell viability. When the $I_{max}$ of at least one out of the four marker genes exceeded the cut-off value of that gene (15-fold for ATF3, 2-fold for DNAJB4 and GCLM, and 4-fold for IL-8) at over 80% cell viability was judged as positive (EpiSensA prediction). Also, the estimated concentration (EC) that showed a fold induction of each cut-off value (ATF3 EC15, DNAJB4 EC2, GCLM EC2 and IL-8 EC4) at over 80% cell viability was calculated using linear interpolation from the values above and below the induction thresholds. If fold inductions at all tested concentrations exceeded the cut-off values, the EC values were calculated by linear extrapolation from the values at the minimum two tested concentrations. $IC_{20}$ (%) NT indicated "Non-Toxic", which means cell viability was over 80% at tested concentration of 100%. If the test chemical exceeded the cut-off value of at least one out of the four marker genes, the chemical was judged as positive (P). If not, the chemical was judged as negative (N). For data from in vitro tests, chemicals judged as positive are indicated as "P", and those judged as negative chemicals are indicated as "N"  DPRA N1; KeratinoSens™ P3; h-CLAT = N1 (Saito et al., 2017).

#### 3.7.3.3 Keratinocyte-based reporter cell line to screen skin sensitisers in vitro

**Study reference:**

Emter R., Ellis G., and Natsch A. (2010): Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicology and Applied Pharmacology 245 (3), 281-290. DOI: 10.1016/j.taap.2010.03.009

**Detailed study summary and results:**

The assay is based on the principle that skin sensitisers are electrophilic molecules and the Nrf2-electrophile-sensing pathway comprising the repressor protein Keap1, the transcription factor Nrf2 and the antioxidant response element (ARE) is emerging as a toxicity pathway induced by skin sensitisers. A luciferase reporter gene under control of a single copy of the ARE-element of the humanAKR1C2 gene was stably inserted into HaCaT keratinocytes in order to routinely test chemicals for significant induction of gene activity of the innate toxicity pathway Keap1-Nrf2-ARE that is induced by most sensitisers (Nrf2 = transcriptional regulator (nuclear factorerythroid2-related factor 2); ARE = antioxidant response element) thus, the luciferase induction for AKR1C2 (coding for analdo-keto reductase) was tested.

Keratinocytes were chosen as test system because they are the first cells which come into contact with compounds applied topically to the skin. Whereas the primary immune cells in the skin are the Langerhans cells, the keratinocytes are also involved in the immune reaction and they are particularly known for their ability to produce a number of cytokines. Chemicals were commercial qualities obtained from Givaudan Schweiz AG, Geneva, Switzerland. The reporter plasmids pGL3-Promoter and pGL4.17 were obtained from Promega (Duebendorf, Switzerland).

A synthetic double-stranded DNA fragment consisting of two complementary oligonucleotides with the sequences 5'-CACTAGTGTGACAAAGCAGCTAGTGTGACAAAGCAGCTAGTGTGACAAAGCAGCT-AGTGTGACAAAGCAG-3' and 5'-GATCTGCTAGCTGCTTTGTCACACTAGCTGCTTTGTCACACT-AGCTGCTTTGTCACACTAGCTGCTTTGTCACACTAGTGGTAC-3' containing four copies of the rat GST2 ARE sequence was synthesised by Microsynth (Balgach, Switzerland).

One to four tandem copies of this sequence were cloned into the KpnI/BglII site of the vector pGL3-Promoter upstream of the SV40 promoter resulting in a luciferase gene under the control of 4, 8, 12 , or 16 tandem copies of the ARE element and the SV40 promoter. In addition, a synthetic double-stranded DNA element consisting of the oligonucleotides 5'-CTGGTCGCAAGGTGTGCAAGCTGCTGAGTCACCCTG-ACTGCATCAACCCCAGGAGCTA-3' and GATCTAGCTCCTGGGGTTGATGCAGTCAGGGTGACTC-AGCAGCTTGCACACCTTGCGACCAGGTAC containing the region around the functional ARE element in the promoter of the human AKR1C2gene was inserted between the KpnI and BglII sites of the vector pGL3-Promoter.

In order to obtain the vector for selecting the stable cell line, the fragment between the restriction sites KpnI and HindIII containing the AKR1C2-ARE insert and the SV40 promoter was excised from the pGL3-based vector and inserted into the KpnI and HindIII sites in the vector pGL4.17. The resulting vector pGL4.17-AKR1C2-ARE-SV40 contains the regulatory construct upstream of the new synthetic version of the luciferase gene luc2, which is codon-optimised for improved expression and which contains a reduced number of consensus transcription factor binding sites as compared to the luciferase gene in pGL3. This new vector also contains a synthetic neomycin phosphotransferase gene for selection of stable clones. Wild-type HaCaT cells were maintained in Dulbecco's modified Eagle's medium containing glutamax (Gibco/Invitrogen) supplemented with 9% fetal calf serum at 37 °C in the presence of 5% $CO_2$.

The medium for the stable engineered cell line KeratinoSens was supplemented with 500 µg/mL G418. For transient transfections and for the generation of the stable cell line KeratinoSens, HaCaT cells were transfected using the Nucleofector®System (Lonza, Switzerland) with the program U-020. For the generation of the stable cell line KeratinoSens, HaCaT cells were transfected with 1 µg of a mix of circular and linearised (BamHI or NotI) plasmid pGL4.17-AKR1C2-ARE-SV40. Stable clones were selected by supplementing the growth medium with 500 µg/mL of G418. Single colonies were isolated, expanded, and frozen. For transient transfection experiments, $10^6$ HaCaT cells were transfected with 2 µg of the respective plasmid and seeded in 96-well plates at $10^4$ cells per well. Twenty-four hours later, fresh medium containing the test chemicals was added to the cells. After 24 h incubation with the test chemicals, the cells were washed once with PBS, lysed using Passive Lysis buffer (Promega, Duebendorf, Switzerland), and the Luciferase activity was determined as described below. Test chemicals were dissolved in DMSO at a concentration of 200 mM. They were serially diluted in DMSO to obtain 12 final concentrations ranging from 0.1 mM to 200 mM. These DMSO solutions were diluted 25-fold in culture medium containing 1 % FCS. The few chemicals not soluble in DMSO were dissolved in $H_2O$, and the DMSO level was adjusted to the same level in the dilutions in cell culture medium.

The KeratinoSens cells were seeded in 96-well plates at a density of 10,000 cells per well in 125 µL growth medium without G418. Medium was replaced after 24 h with 150 µl fresh medium containing only 1% of FCS. FCS medium (1%) containing the different dilutions of the DMSO solutions were added to the different wells (50 µL). Final solvent concentration was thus 1% and test concentrations for each chemical ranged from 1 µM to 2,000 µM. In each experiment, testing was done in triplicate. As a control tert-butyl-hydroquinone was always included in each test plate, and each plate contained six control wells with cells and solvent. In parallel all chemicals were tested for cytotoxicity with the MTT reduction test in a parallel plate in each repetition. All the plates were covered with a foil (Sealing tape SI, Nunc). After 48 h incubation with the test chemicals, the medium was removed and cells were washed once with PBS. To each well, 20 µL of passive lysis buffer (Promega, Duebendorf, Switzerland) was added and the cells were incubated for 20 min at RT. Plates were then read in a Promega Glomax luminometer with automatic injection of 50 µL of the luciferase substrate to each well and integration of the luciferase activity for 2s.

For the cell viability assay, 27 µl of a MTT solution (5 mg/mL in DPBS) was added to each well. After 4 h incubation, the medium was removed and 200 µl of a 10% SDS solution was added to each well. After the cells have dissolved completely, the absorption at 600 nm was determined for each well. All tests were repeated at least twice with triplicate analysis. Based on these experiments, for from a promoter known to be induced by skin sensitisers appears to be a better choice in a keratinocyte background. Nine stable, recombinant HaCaT clones based on the plasmidpGL4.17-AKR1C2-ARE-SV40 were tested.

For each clone the absolute light output and the dynamic range of luciferase induction by sensitisers were evaluated. Clone 8 was selected based on the following criteria: (i) best signal to noise ratio and (ii) highest dynamic range if treated with the weak sensitisers Lyral and benzyl salicylate called "KeratinoSens" was used for the further development of a standard operating procedure (SOP). Cell number at seeding was varied between 5,000 and 20,000 cells per well in 96-well plates, incubation time with the chemicals was varied between 7 and 48 h, solvent concentration was varied between 0.06 and 1% DMSO and the serum level was varied between 0 and 10%. While the incubation time of 48 h with of 10,000 cells per well was found optimal. Testing chemicals at too high concentrations may in some cases lead to false-positive results either due to (i) unspecific induction of stress response or (ii) contaminants in the test chemical preparation. Cytotoxicity of a compound is not a prerequisite for a positive result in the test and the EC1.5 and the IC50 are not closely linked for most chemicals. This assay may predict the hazard for a wide variety of chemical classes of structurally diverse sensitisers and non-sensitisers, yet some prohaptens may remain undetected chemicals with a unique reactivity toward amine-groups such as anhydrides do not induce the Nrf2-pathway. Nevertheless, benzyl salicylate was positive in the KeratinoSens assay (Emter et al., 2010).

#### 3.7.3.4 Feasibility study to support a threshold of sensitisation concern concept based on human data

**Study reference:**

Keller D., Krauledat M., and Scheel J. (2009): Feasibility study to support a threshold of sensitization concern concept in risk assessment based on human data. Archives of Toxicology 83 (12), 1049-1060. DOI: 10.1007/s00204-009-0460-9

**Detailed study summary and results:**

In analogy to the Threshold of Toxicological Concern concept, a Threshold of Sensitisation Concern (TSC) concept is proposed for chemicals with respect to their ability to induce an allergic contact dermatitis. Previously, the derivation of a dermal sensitisation threshold was suggested based on an evaluation of animal data, now the human data were considered upon performing a meta-analysis taking into account No Expected Sensitisation Induction Levels for fragrance ingredients from the IFRA/RIFM dataset. Based on a statistical analysis by applying Sensitisation Assessment Factors that account for inter-individual variability and different exposure conditions, TSC values of 0.91 or 0.30 µg/cm$^2$ can be derived in terms of amount per skin area. TSC values are compared with typical exposure levels of cosmetic products. The authors conclude that substances with a quotient of exposure level and TSC < 1 can be considered "virtually safe" (Keller et al., 2009).

### 3.7.3.5 Induction of antioxidant response element-dependent genes

**Study reference:**

Natsch A. and Emter R. (2008): Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. Toxicological Sciences 102 (1), 110-119. DOI: 10.1093/toxsci/kfm259

**Detailed study summary and results:**

Two model systems: (1) the ARE-regulated quinone reductase (QR) activity in Hepa1C1C7 cells and (2) the ARE-regulated luciferase activity in the cell line AREc32, which contains an eightfold repeat of the ARE sequence upstream of a luciferase reporter gene were used to test the activation of the Keap/Nrf2/ARE regulatory pathway (102 different chemicals; commercial qualities obtained from Givaudan Schweiz AG, Geneva, Switzerland) by chemicals with known skin sensitisation potential. AREc32 is a stable cell line derived from the human MCF7 breast carcinoma cell line (licensed from CRX biosciences, Dundee, Scotland); maintained in Dulbecco's modified Eagle's medium containing glutamax (Gibco/Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum and 500 µg/mL G418. Hepa1C1C7 cells were obtained from ATCC (European distributor, LGC Promochem, France) and were cultured in Dulbecco's modified Eagle's medium without nucleotides and deoxynucleotides (Gibco/Invitrogen) supplemented with 10% fetal calf serum. Both cell lines were grown at 37 °C in the presence of 5% CO2.

Test chemicals were dissolved in acetonitrile or dimethyl sulfoxide (DMSO) at a concentration of 100 mM, further diluted in culture medium to a final concentration of either 10 or 2.5 mM, and then serially diluted in culture medium containing an equal concentration of solvent. AREc32 cells were seeded in 96-well plates at a density of 50,000 cells per well in 180 µL of growth medium. Test chemicals were added 40 h later dissolved in 20 µL of growth medium. Final solvent concentration was 0.25% in all experiments, unless a concentration range up to 1,000 µM was tested: in this case solvent levels were at 1%.

After 24 h of exposure, cells were washed with phosphate buffered saline (PBS) without $Ca^{2+}$ and $Mg^{2+}$ and then lysed by the addition of 20 µL of passive lysis buffer (Promega AG, Wallisellen, Switzerland). Luciferase activity was initiated by adding 50 µL of the luciferase assay substrate dissolved in luciferase assay buffer (both from Promega) to the cell lysate. Alternatively, 50 µL of assay reagent were made up according to the following recipe: 20 mM tricine; 2.67 mM $MgSO_4$; 0.1 mM ethylenediaminetetraaceticacid; 33.3 mM dithiotreitol; 270 µM coenzyme A; 470 µM luciferin potassium salt (Synchem, Kassel, Germany); 530 µM adenosine triphosphate; pH 7.8. Luciferase activity was measured with the GloMax luminometer (Promega). Hepa1C1C7 cells were seeded in 96-well plates at a density of 50,000 cells per well and treated with test chemicals as described for the AREc32 cells. 24 h after addition of the compounds, the QR activity was determined. Cells were lysed by addition of a digitonin solution. A reaction mixture was added, which contained menadione as a QR substrate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP+ (oxidised nicotinamide adenine dinucleotidephosphate), and flavin adenine dinucleotide (oxidised) as electron donating system and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide(MTT), bovine serum albumin, Tween20, and a Tris-buffer. QR reduces menadione to menadiol and the QR-specific activity is determined by measuring the NADPH dependent, menadiol-mediated reduction of MTT to a blue formazan dye. In both the QR and the luciferase assays, tertbutyl-hydroquinone was included as a positive reference chemical in each assay plate.

Cytotoxicity of the compounds for both cell lines was tested in parallel assays run under equal conditions and with equal test concentrations. Twenty-four hours after test chemical addition 27 µL of a 5 mg/mL solution of MTT in PBS was added to the growth medium, cells were incubated for further 4 h at 37 °C and then the growth medium was discarded. Cells were lysed for 24 h by the addition of 200 µL of 10% sodium dodecyl sulfate (SDS), and then the optical density of the reduced formazan dye was measured at 600nm. Data are expressed as IC50 values (inhibitory concentration reducing viability by 50%).The screening on the AREc32 cell line was repeated three or four times, with duplicate analysis for each chemical at each test concentration in each repetition. In the first two repetitions four concentrations (2, 10, 50, and 250 µM) were tested. In the third and fourth repetition, six binary dilutions covering the maximal non cytotoxic doses for each test chemical were selected. Wherever possible, tests up to a maximal dose of 1000 µM were performed in these repetitions.

For chemicals with contradictory results, further repetitions were made to clarify whether they are indeed ARE-inducers or not. The screening with the Hepa1C1C7 cell line was repeated twice, with duplicate analysis at four concentrations (2, 10, 50, and 250 µM) in each experiment. Based on these experiments, for each test chemical (1) the average maximal induction of gene activity ($I_{max}$), (2) the concentration range for maximal induction ($CI_{max}$), and (3) the average concentration inducing significantly enhanced gene activity above a certain threshold (EC1.25 for QR and EC1.5 for luciferase activity) were determined. The latter calculations were performed with log-linear extrapolation from the values above and below the induction threshold (as for the EC3 value determination in the LLNA).

A chemical was rated positive, if it induced significantly enhanced gene activity above the threshold indicated above at any of the tested concentrations and either in all repetitions made or in three out of four or four out of five repetitions. LLNA data were determined under standard conditions as defined in the OECD guideline 429; further LLNA data were either taken from the general literature or from the Research Institute on Fragrance Materials RIFM. To rate the chemicals, EC3 values were expressed in millimolar to give a better comparison between chemicals. The results show for benzyl salicylate that the EC3 value in the LLNA is 127.19 mM, leading to the assignment of the sensitisation class "Moderate" based on the LLNA.

For benzyl salicylate, cytotoxicity (IC50) which represents the concentration reducing cell viability of AREc32 by 50% after 24 h as measured with the MTT assay was > 1000 mM. Benzyl salicylate was able to induce the ARE-regulated luciferase activity resulting in an $I_{max}$ of 3.6 and a $CI_{max}$ of 250, while the EC1.5 was 18.1. The results are the outcome of 3 out of 3 repetitions with significant luciferase induction/number of repetitions made and as a consequence, benzyl salicylate was rated positive in this study (Natsch and Emter, 2008).

### 3.7.3.6 Battery approach involving data from different *in vitro* and *in silico* assays

**Study reference:**

Natsch A., Emter R., and Ellis G. (2009): Filling the concept with data: Integrating data from different *in vitro* and *in silico* assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicological Sciences 107 (1), 106-121. DOI: 10.1093/toxsci/kfn204

**Detailed study summary and results:**

Literature data of known skin sensitisation potential chemical have been integrated on the basis of the following parameters: (1) peptide reactivity as a surrogate for protein binding, (2) induction of antioxidant/electrophile responsive element dependent luciferase activity as a cell-based assay; (3) Tissue Metabolism Simulator skin sensitisation model in silico prediction; and (4) calculated octanol-water partition coefficient. The results of the *in vitro* assays were scaled into five classes from 0 to 4 to give an in vitro score and compared to the local lymph node assay (LLNA) data, which were also scaled from 0 to 4 (non-sensitiser/weak/moderate/strong/extreme). Different ways of evaluating these data have been assessed to rate the hazard of chemicals (Cooper statistics) and to also scale their potency. With the optimised model an overall accuracy for predicting sensitisers of 87.9% was obtained. There is a linear correlation between the LLNA score and the in vitro score. However, the correlation needs further improvement as there was still a relatively high variation in the *in vitro* score between chemicals belonging to the same sensitisation potency class.

All fragrance chemicals were commercial qualities obtained from Givaudan Schweiz AG, Geneva, Switzerland. All other test chemicals were purchased from Fluka/Sigma/Aldrich, Buchs, Switzerland. LLNA data have all been published previously. AREc32 is a stable cell line derived from the human MCF7 breast carcinoma cell line which has been licensed from CRX biosciences, Dundee, UK. AREc32 cells were maintained, prepared for the test, treated with chemicals and assayed for luciferase activity. The screening of the chemicals not contained in a previous publication (Natsch and Emter, 2008) was repeated four times, with duplicate analysis for each chemical at each test concentration in each repetition and with six binary dilutions covering the maximal non-cytotoxic doses for each test chemical. Based on these experiments, for each test chemical (1) the average maximal induction of gene activity ($I_{max}$; reported as fold-induction vs. untreated cells) and (2) the average concentration inducing 1.5-fold enhanced gene activity (EC 1.5; reported

in μM) were determined. The latter calculations were performed with log-linear extrapolation from the values above and below the induction threshold (as for the EC3 value determination in the LLNA).

A chemical was rated positive, if it induced significantly enhanced gene activity above the threshold at any of the tested concentrations either in all repetitions made or in three out of four repetitions. The literature data are based on three or four repetitions with duplicate analysis in each repetition. Peptide reactivity with the Cys-containing peptide Ac-RFAACAA was determined. Further peptide reactivity data were taken from the publications. Computer modeling and statistics: cLogP values were obtained either from internal data (measured values according to OECD guideline 117) or calculated using KOWWIN V.1.67 obtained from the United States Environmental Protection Agency (U.S. EPA) web site. The TIMES SS software (V.2.25.7) was obtained from OASIS Laboratory of Mathematical Chemistry, Bourgas, Bulgaria, and run using the skin sensitisation metabolism activated toxicity model. Regression analysis and plotting of Box plots were performed with the Minitab statistical software (Minitab Inc., version 15.1.1.0, Coventry, UK).

For benzyl salicylate, ARE concurred with the positive LLNA result, while based on peptide reactivity as well as on the TIMES-SS software, a false negative prediction was obtained (Natsch et al., 2009).

### 3.8 Germ cell mutagenicity

Not evaluated for this dossier

### 3.9 Carcinogenicity

Not evaluated for this dossier

### 3.10 Reproductive toxicity

Not evaluated for this dossier

### 3.11 Specific target organ toxicity – single exposure

Not evaluated for this dossier

### 3.12 Specific target organ toxicity – repeated exposure

Not evaluated for this dossier

### 3.13 Aspiration hazard

Not evaluated for this dossier

## 4 ENVIRONMENTAL HAZARDS

Not evaluated for this dossier

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