

Assessment of the skin sensitization potential of fragrance ingredients using the U-SENSTM assay

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ABSTRACT

The U-SENSTM assay was developed to address the third key event of the skin sensitization adverse outcome pathway (AOP) and is described in OECD test guideline 442E, Annex II. A dataset of 68 fragrance ingredients comprised of 7 non-sensitizers and 61 sensitizers was tested in the U-SENSTM assay. The potential for fragrance ingredients to activate dendritic cells, measured by U-SENSTM, was compared to the sensitization potential determined by weight of evidence (WoE) from historical data. Of the non-sensitizers, 4 induced CD86 cell surface marker ≥ 1.5 -fold while 3 did not. Of the sensitizers, 50 were predicted to be positive in U-SENSTM, while the remaining 11 were negative. Positive and negative predictive values (PPV and NPV) of U-SENSTM were 93% and 21%, respectively. No specific chemical property evaluated could account for misclassified ingredients. Assessment of parent and metabolite protein binding alerts *in silico* suggests that parent chemical metabolism may play a role in CD86 activation in U-SENSTM. Combining the U-SENSTM assay in a "2 out of 3" defined approach with the direct peptide reactivity assay (DPRA) and KeratinoSensTM predicted sensitization hazard with PPV and NPV of 97% and 24%, respectively. Combining complementary *in silico* and *in vitro* methods to the U-SENSTM assay should be integrated to define the hazard classification of fragrance ingredients, since a single NAM cannot replace animal-based methods.

1. Introduction

The development of non-animal alternatives for safety assessment has been a priority of toxicologists for years (Kleinstreuer et al., 2018a). The push to develop new approach methodologies (NAMs), is necessitated by several factors, including ethical concerns, change in societal outlook, a general desire to explore new scientific innovations, and the European Union's (EU's) legislative ban of animal testing for cosmetic ingredients (Leist et al., 2014; Pistollato et al., 2021). The skin sensitization toxicological endpoint has particularly seen considerable investments in the development of NAMs, with hazard identification and characterization progressing from the use of guinea pigs through the refined use of the validated murine local lymph node assay (LLNA) to the present, where several *in silico*, *in chemico*, and *in vitro* alternatives have been developed (Adler et al., 2011; Del Bufalo et al., 2018; Reisinger et al., 2015).

The development and validation of non-animal alternatives for skin

sensitization assessment have been especially successful, primarily due to formalization of the adverse outcome pathway (AOP) (OECD, 2014). The skin sensitization AOP characterizes the mechanisms involved in allergic contact dermatitis in four defined key events. Several *in chemico* and *in vitro* methods developed based on these key events have been validated and adopted in test guidelines by the Organization for Economic Co-operation and Development (OECD). The direct peptide reactivity assay (DPRA; OECD TG 442C) assesses chemical reactivity to skin proteins in key event 1 (OECD, 2020b), KeratinoSensTM and LuSens (OECD TG 442D) assess keratinocyte activation in key event 2 (OECD, 2018a, 2018b), while the human cell line activation test (h-CLAT) and U937 cell line activation test (U-SENSTM) (OECD TG 442E) evaluate dendritic cell activation in key event 3 (OECD, 2018a, 2018b). Currently, *in vitro* methodologies are yet to be developed to assess key event 4 of skin sensitization, lymphocyte activation. All these *in vitro* assays are single-point tests providing limited mechanistic information; therefore, none of them have been endorsed as standalone tests to

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replace the gold standard LLNA.

The U-SENSTM assay addresses the third key event of skin sensitization by quantifying the change in the expression of a cell surface marker associated with the process of activation of monocytes and DC (*i.e.*, CD86), in the human histiocytic lymphoma cell line U937, following exposure to potential sensitizers. The measured expression levels of CD86 cell surface marker in the U937 cell line is then used for supporting the discrimination between skin sensitizers and non-sensitizers.

Defined approaches have been proposed and evaluated to integrate currently validated *in vitro* methods to identify and characterize the sensitization potential of chemicals (Kleinstreuer et al., 2018b; OECD, 2019; Strickland et al., 2016; Strickland et al., 2017; Zang et al., 2017). Diverse chemical classes must be tested to understand the applicability domain of the new *in vitro* methods and provide robust data for integration into the DAs. Fragrance ingredients are generally structurally simple, low molecular weight, predominantly semi-volatile substances consisting of carbon, hydrogen, and oxygen. We tested fragrance ingredients in the U-SENSTM assay. This set of fragrance ingredients was selected because these chemicals had sufficient and robust historical human and animal data to determine skin sensitization hazard. Additionally, *in vitro* studies were also available, addressing key events 1, 2, and 3 of the skin sensitization AOP for most of the fragrance ingredients in this dataset. We evaluated how positive predictions from the U-SENSTM assay compared to the sensitization potential of fragrance ingredients determined by weight of evidence (WoE) from historical *in vivo* and *in vitro* data and from methods covering key events 1, 2, and 3. Physico-chemico properties were also evaluated to determine whether they influenced the outcomes of the U-SENSTM assay. Further analysis was performed using the OECD Toolbox and Toxtree *in silico* tools to determine how protein binding alerts correlate with U-SENSTM predictions for this fragrance ingredient data-set.

2. Materials and methods

2.1. The dataset

The chemicals tested in the U-SENSTM assay included 68 fragrance ingredients with sufficient historical human and/or animal data to determine their sensitization hazard and potency by WoE, as previously described (Api et al., 2015) (Na et al., 2021, accepted for publication in *Dermatitis*). Historical data, including human, animal, and *in vitro* studies on the test chemicals, were collated from the Research Institute of Fragrance Ingredients Inc. (RIFM) database (<https://rifmdatabase.rifm.org>) and other publicly available sources such as the European Chemicals Agency (ECHA; <https://echa.europa.eu/>). All measured physico-chemical data such as octanol/water partition coefficient, log K_{OW}, were obtained from the RIFM database. When measured log K_{OW} data were unavailable, the values were estimated using the Estimation Programs Interface SuiteTM (EPI SuiteTM), a Windows®-based set of physical/chemical property and environmental fate estimation programs developed to screen new chemicals lacking experimental data (EPA, 2012).

Seven chemicals had sufficient animal data to be considered non-sensitizers, meaning that no positive animal or human data were found on these chemicals, and the data obtained were negative in the LLNA, guinea pig studies, and/or confirmation of no induction in humans (CNIH) studies (patch tests). All 7 non-sensitizers had negative LLNA data when tested between 25% and 100%, and 2 of the non-sensitizers were also negative in guinea pig studies conducted according to OECD guidelines. Additionally, the 7 non-sensitizers had been tested in CNIH studies that confirmed the negative results obtained in the LLNA. The remaining 61 materials in the dataset had been evaluated to be sensitizers with WoE no expected sensitization induction levels (NESILs) spread over a wide range from 110 µg/cm² to 47,000 µg/cm². Most of the fragrance ingredients in the dataset were evaluated to be sensitizers due to positive LLNA, guinea pig, and/or human data.

Thirteen of the sensitizers were not positive in the LLNA but had been evaluated to be sensitizers based only on guinea pig or human data. Six fragrance ingredients included in this data set were natural complex substances (NCS) and all had been evaluated to be sensitizers based on RIFM's WoE approach.

Below are general protocols for how historical experiments were conducted on the dataset of 68 fragrance ingredients.

2.2. Human studies

2.2.1. Confirmation of no induction in humans (CNIH)

CNIH studies were conducted on male and female volunteer subjects to evaluate the dermal sensitization of test chemicals. Occlusive patches were used throughout the studies. For the induction, the test chemical in vehicle (typically ethanol or 1:3 ethanol:diethyl phthalate) was applied to each designated patch on the back for 24 h, generally on a Monday, Wednesday, and Friday schedule. The identical patch site was re-patched until 9 such induction patchings were completed over the course of 3 weeks. A 2-week rest period, during which no material was applied, followed the final induction patching. During the challenge phase, the patches identical to the induction patches were applied to previously untreated sites on the back and remained on for 24 h. The challenge sites were scored 24, 48, 72, and sometimes 96 h post patch application. CNIH studies were generally conducted to confirm the no observed effect level (NOEL). NOELs were considered sufficient to set the NESIL when they were conducted according to RIFM's standard protocol (Politano and Api, 2008).

2.2.2. Human maximization test (HMT)

In the HMTs, test materials were generally applied in petrolatum under occlusion on the same site of the volar aspects of the forearms of subjects for 5 alternate-day, 48-h periods. Patch sites were pretreated for 24 h with 5%–10% aqueous sodium lauryl sulfate (SLS) under occlusion. Following a 10- to 14-day rest period, challenge patches were applied under occlusion to fresh sites for 48 h. Challenge applications were preceded by 30- to 60-min applications of 5%–10% aqueous SLS under occlusion. Challenge scoring occurred upon patch removal and 24 h thereafter (Kligman, 1966).

2.3. Animal studies

2.3.1. The local lymph node assay (LLNA)

LLNAs were generally conducted according to OECD 429 and Good Laboratory Practices guidelines (OECD, 2010). Treated groups of animals received the test material at a range of concentration dilutions. A negative control group of animals received the vehicle. A positive control group was treated concurrently, or a separate reliability check was conducted regularly with a known sensitizer. During the induction phase, 25 µL of the test material, vehicle, or positive control were applied to each ear for 3 consecutive days. Following 2 days of rest, each animal received a single intravenous injection of 250 µL of saline containing 20 µCi of ³H-TdR. Approximately 5 h later, auricular lymph nodes were excised, and lymphocyte proliferation was quantified by beta scintillation counting. The Stimulation Index (SI) was obtained by calculating the ratio of disintegrations per minute (dpm) of the treated group divided by the dpm of the vehicle control group. When the SIs from the range of concentrations was less than a threshold of 3, the test chemical was not considered to be sensitizing up to the maximum concentration tested. Chemicals were classified as skin sensitizers if, at any test concentration (up to and including 100%), they induced a stimulation index of 3 or more compared with the concurrent vehicle control, along with consideration of dose-response. The EC3 (concentration required to elicit an SI value greater than or equal to 3) was estimated by linear interpolation for sensitizers or log-linear extrapolation using the two lowest test concentrations from the dose response curve (Ryan et al., 2007). When multiple EC3 values were available for a

chemical, the average was calculated.

2.3.2. The guinea pig maximization test (GPMT)

GPMTs were generally conducted in guinea pigs according to the method described by Magnusson and Kligman, following OECD guidelines (OECD, 1992). Intradermal injection and topical application concentrations were typically determined during the preliminary dose range finding studies. The main study consisted of an induction phase and a challenge phase. For the induction phase, 3 pairs of 0.1 mL intradermal injections were made simultaneously into a shoulder area. Injections for the test group animals were as follows: 1) 1:1 Freund's complete adjuvant in water, 2) Test chemical diluted in vehicle, and 3) Test chemical in vehicle in 50:50 mixture with Freund's complete adjuvant. The control group animals were treated similarly but without the test material. One week after the injections, the same area was clipped free of hair and the test chemical diluted in vehicle was applied over the clipped area. This patch was covered with an occlusive patch and left in place for 48 h. Two weeks later, the animals were challenged topically with the test chemical in vehicle. For the challenge, the material was typically applied in the same manner as topical induction application. The patch was occluded and left for 24 h. The challenge site was evaluated 24, 48, and 72 h after patch application. Chemicals were classified as non-sensitizers or sensitizers (strong, moderate, or weak) according to the ECETOC Technical Report 87 (ECETOC, 2003).

2.3.3. The Buehler test

Buehler delayed contact hypersensitivity studies were conducted on guinea pigs generally in accordance with OECD Guideline 406 (OECD, 1992). Typically, an aliquot of the test chemical in vehicle was applied for approximately 6 h under occlusion. The applications were repeated once per week for 3 consecutive weeks, and a total of 3 applications were made. After a 2-week rest period, challenge applications were made to untreated skin sites with a concentration of the test chemical. The reactions were assessed at 24 and 48 h after the dermal treatment. Chemicals were classified as non-sensitizers or sensitizers (strong, moderate, or weak) according to the ECETOC Technical Report 87 (ECETOC, 2003).

2.4. In chemico and in vitro studies

2.4.1. The direct peptide reactivity assay (DPRA)

The *in chemico* DPRA evaluates activation of the first key event of the AOP. This assay measures the reactivity of chemicals towards cysteine- and lysine-containing peptides. The DPRA has been validated and formally adopted by the OECD as Testing Guideline 442C (OECD, 2020). Generally, the mean peptide depletion is quantified after a 24-h incubation of the test chemicals with cysteine- and lysine-containing peptides. Chemicals with mean cysteine and lysine peptide reactivity <6.37% are assigned no or minimal reactivity, while 6.37% to 22.62%, 22.62% to 42.27%, and above 42.47% are assigned low, moderate, and high reactivity, respectively (OECD, 2020b). Minimally reactive chemicals are classified as negative while the other reactivity groups are regarded positive for sensitization. Sometimes, the results were considered inconclusive, due to the co-elution of both synthetic peptides and the test chemical.

2.4.2. The KeratinoSens™ assay

This assay measures activation of the second key event of the skin sensitization AOP. KeratinoSens™ measures keratinocyte activation using the luciferase reporter gene activation as a surrogate for Nrf2-mediated activation of antioxidant response element (ARE). KeratinoSens™ has been validated and adopted by the OECD as Testing Guideline 442D (OECD, 2018). Generally, cells are treated with concentrations of the test article ranging from 0.98 μ M to 2 mM in three replicate 96-well plates, then luciferase activity and cytotoxicity are quantified after 48 h of incubation. Test chemicals with an induction of

the luciferase activity of more than 1.5-fold while maintaining at least 70% viability in a minimum of 2 experiments are considered positive (OECD, 2018b).

2.4.3. The human cell line activation test (h-CLAT)

The h-CLAT evaluates dendritic cell activation, the third key event of the skin sensitization AOP. This assay has been validated by the OECD and adopted as Test Guideline 442E annex I (OECD, 2018b). Induction of cell surface markers CD54 and CD86 is quantified in human monocytic leukemia cells (dendritic cell surrogate) after 24-h incubation with the test chemical. A 2-fold induction of the CD54 expression and/or 1.50-fold induction of CD86 expression at cell viabilities of at least 50% is rated positive for dendritic cell activating potential of a test substance (OECD, 2018a).

2.5. In silico predictions

In silico protein binding alerts were generated in the OECD Toolbox v 4.2 and Toxtree v. 3.1.0 software. Fragrance ingredient CAS numbers and SMILES were imported into the OECD Toolbox v 4.2 software in a batch run using the "Import as inventory" function. The following profiling methods were then selected: "Protein binding alerts for skin sensitization by OASIS," "Protein binding alerts for skin sensitization by OASIS, with Autooxidation simulator," and "Protein binding alerts for skin sensitization by OASIS, with Skin metabolism simulator". A description on how to download and use the OECD toolbox software is detailed in the end-user manual (OECD, 2017). Batch processing mode was used in Toxtree v 3.1.0 for target chemical protein binding alerts. "Skin sensitization reactivity domains" was selected under "Decision tree domain" which is located under the "Methods" tab of the software. Details on how the Toxtree software is used to generate skin sensitization protein binding alerts are provided at Toxtree – Toxtree Module: Skin sensitisation reactivity domains (sourceforge.net).

2.6. U-SENS™ assay

2.6.1. Cell culture

U937 human monocyte (ATCC no. CRL-1593.2TM) sourced from the ATCC (American Type Culture Collection, Virginia, USA) were used following an acclimatization period of approximately 8 days after thawing and were not sub-cultured more than 21 times. The cultures were checked for mycoplasma contamination. Stock and treatment cultures were performed in RPMI 1640 medium (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% (v/v) heat-inactivated (56 °C; 30 min) fetal calf serum (FCS; Life Technologies), L-glutamine (2 mM; Life Technologies), and penicillin/streptomycin (50 U/mL and 50 μ g/mL respectively; Life Technologies). All incubations were carried out in a humid atmosphere (80–100%) containing 5.0 $\pm$ 0.5% CO₂ in air in the dark at 37.0 $\pm$ 1.0 °C.

2.6.2. U-SENS™ assay

A solubility test was performed on test chemicals prior to testing. The chemicals were dissolved or stably dispersed at a concentration of 50 mg/mL in either complete RPMI 1640 medium or dimethyl sulfoxide (DMSO; Sigma, Zwijndrecht, The Netherlands). The order of preference being complete RPMI 1640 medium and then DMSO. Eventually, except for 4 chemicals that were dissolved in RPMI, jasmine Absolute (Sambac), ylang ylang, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, and longifolene, all chemicals were dissolved in DMSO.

The U-SENS™ was performed according to OECD test guideline 442E annex II (OECD, 2018). U937 human monocytes were exposed to the test substances for 45 $\pm$ 3 h. After incubation the cells were stained with propidium iodide (0.5 μ g/mL; Sigma, Zwijndrecht, The Netherlands) and fluorescein isothiocyanate-conjugated CD86 antibodies (#555657; BD Pharmingen, Amsterdam, The Netherlands) or mouse IgG1 antibodies (isotype control; #555748; BD Pharmingen, Amsterdam, The

Netherlands). The propidium iodide staining was used to assess the viability for each of the test item concentrations. The acquisition was performed with a BD FACSCanto flow cytometer and further analyzed with BD FACSDiva software v 5.0.3. For test item concentrations with a viability of at least 70%, the CD86 stimulation index (S.I.) was calculated relative to the corresponding solvent control (medium or 0.4% DMSO). If applicable, the EC150 (concentration with an S.I. of 150%) value was calculated by linear interpolation. At least two valid independent runs were performed. In the first run, 6 fixed concentrations were tested while in the subsequent run(s) the test item concentrations were adapted, only if necessary, based on the cytotoxicity and CD86 S.I. of the test item.

The U-SENSTM assay is regarded as negative if two independent runs result in an S.I. of CD86 lower than 150% with a cell viability $\geq 70\%$. The U-SENSTM assay is regarded as positive if two independent runs result in an S.I. of CD86 higher or equal to 150% with a cell viability $\geq 70\%$. However, if, in the first run, the S.I. of CD86 is higher or equal to 150% at the highest non-cytotoxic concentration only, the run is not conclusive, and additional concentrations between the highest non cytotoxic concentration and the lowest cytotoxic concentration were tested in at least two additional runs. If precipitation or color interference was observed - but no induction -, the assay was also classified as positive following the guideline.

3. Results

All the historical data and U-SENSTM results are summarized in Table 1.

3.1. Predictive capacity of U-SENSTM for fragrance ingredients

In the set of non-sensitizers, 3 were negative while 4 were positive in U-SENSTM. All 7 non-sensitizers had been found to be negative in the LLNA, but only 2 had been tested to the maximum concentration of 100% (Fig. 1). It could not be concluded whether any of the other 5 chemicals would be sensitizing if tested at higher concentrations in the LLNA, which would possibly explain the positive results observed in U-SENSTM results. Within the set of 61 sensitizers, 50 were predicted to be positive while the remaining 11 gave false negative results, leading to an 18% false negative rate. Positive predictions were observed for a wide range of sensitizer potencies, with NESILs ranging from 110 $\mu\text{g}/\text{cm}^2$ to 47,000 $\mu\text{g}/\text{cm}^2$ (Fig. 2). U-SENSTM predicted the sensitization potential of this set of fragrance ingredients with a sensitivity, specificity, and accuracy of 82%, 43%, and 78%, respectively. Additionally, positive, and negative predictive values (PPV and NPV) were assessed to be 93% and 21%, respectively, when U-SENSTM predictions were compared to assessments from weight of evidence from *in vivo* data (combination of historical human and animal data). Comparing U-SENS just to LLNA data yielded a PPV of 70% and NPV of 50% (Table 1). With focus on false negative and false positive predictions, no specific chemical property evaluated (e.g., log K_{ow} , volatility, solubility, and interference) could account for the misclassified ingredients.

3.2. U-SENSTM vs. *in silico* prediction analysis

Protein binding alerts were obtained from the OECD Toolbox V4.3 and Toxtree 3.1.0 softwares. *In silico* protein binding alerts found for this fragrance ingredient dataset included Michael addition, Schiff base formation, acylation, SN2, and unspecified nucleophilic addition. No *in silico* predictions were available for the 6 NCS in the dataset because each constitutes a complex mixture of discrete chemicals. Parent chemical protein binding alerts were found in the OECD Toolbox for only 31 of the 62 discrete fragrance ingredients, but 6 of the remaining had alerts in Toxtree. Metabolite protein binding alerts were found for 17 of the 31 fragrance ingredients without parent binding alerts in Toolbox, resulting in a total of 54 protein alerts for the 62 discrete

chemicals. Only 27 of the 48 discrete chemicals predicted to be positive in U-SENSTM had parent protein binding alerts (Fig. 3, pie chart). However, 13 of the remaining chemicals that were positive in U-SENSTM also generated metabolite protein binding alerts (Fig. 3, Bar graph). Four of the 14 chemicals predicted to be negative in U-SENSTM had parent binding alerts (Schiff base and SN2) while the remaining 10 had none.

3.3. U-SENSTM in context of other *in vitro* assays in the skin sensitization AOP

Most of the fragrance ingredients in this dataset also had *in vitro* data from assays evaluating key events 1, 2, and 3 of the skin sensitization AOP (Table 1 and Fig. 4). 67 chemicals in the dataset had been tested in the DPRA. In the DPRA, 5 fragrance ingredients were found to be inconclusive, primarily due to coelution of the test chemicals with the cysteine or lysine peptides; 37 were negative with mean cysteine and lysine peptide depletion between 0% and 6.38% (minimal reactivity); and 25 were positive with mean cysteine and lysine peptide depletion between 6.39% and 100% (ranging from low to high reactivity). There was a 52% concordance between U-SENSTM and DPRA predictions, with 32 matches out of 62 chemicals with conclusive results. All the 68 chemicals had conclusive results from the KeratinoSensTM assay, of which 34 were predicted to be negative while the remaining 34 were positive. A 47% concordance was observed between U-SENSTM and KeratinoSensTM predictions, with 32 out of the 68 matching. Conclusive data were also available for the h-CLAT assay on 65 of the materials in the dataset, and there was an 82% match between U-SENSTM and h-CLAT predictions (53 out of 65). While U-SENSTM had PPV and NPV of 93% and 21%, respectively when compared to WoE hazard assessment from historical data, both values were lower when similarly assessed in the DPRA (PPV = 76%; NPV = 18%), KeratinoSensTM (PPV = 79%; NPV = 16%) and h-CLAT (PPV = 89%; NPV = 0%).

Among the non-sensitizers, a concordance of 57% (4 out of 7), 29% (2 out of 7), and 57% (4 out of 7) was observed when U-SENSTM was compared to DPRA, KeratinoSensTM, and h-CLAT results, respectively. For the sensitizers, the concordance was 50% (27 out of 54), 50% (27 out of 54), and 86% (49 out of 57), respectively, when U-SENSTM was compared to the same key events. For non-sensitizers and sensitizers, a 70% (5 out of 7) and 50% (27 out of 54) match, respectively, was observed between DPRA and KeratinoSensTM results. The PPV and NPV for this set of 32 materials with concordance in DPRA and KeratinoSensTM was 100% and 26%, respectively. When tested in the 2 out of 3 *in vitro* defined approach, 97% PPV and 24% NPV were obtained when U-SENSTM was considered in combination with the DPRA and KeratinoSensTM test method for 62 fragrance ingredients with conclusive results (Table 2).

4. Discussion

Skin sensitization is one of the key toxicological endpoints evaluated in safety assessments on fragrance ingredients. A combination of incentives, including ethical concerns and the EU's legislative ban of animal testing on cosmetic ingredients and products, have accelerated the innovation of NAMs employed in the safety assessment of cosmetics. For skin sensitization, NAMs have primarily been developed based on the skin sensitization AOP. U-SENSTM was developed to address the third key event of the AOP by measuring the expression of the CD86 cell surface marker in U937, histiocytic lymphoma cells, using flow cytometry. This assay quantifies the change in the expression of CD86, and stimulation indexes (SIs) are calculated as the ratio of CD86+ cells of the test group and the control group. SIs $\geq 150\%$ are considered positive.

In this study, a set of 68 fragrance ingredients with sufficient historical human and animal data to determine sensitization potential, by a WoE approach, were tested in the U-SENSTM assay. The predictions from U-SENSTM were then compared to the WoE conclusions from a combination of historical *in vivo* data (animal and human data). U-SENSTM

Table 1
Summary of U-SENS™ results and historical *in vivo* and *in vitro* data.

		U-SENS™ RESULTS			HISTORICAL DATA									
Name	CASRN	Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% µg/cm ²)	CNIH (µg/cm ²)	HMT (µg/cm ²)	GPMT or Buehler	DPRA	KS	H-CLAT
A. Non-sensitizers														
Isobornyl acetate	125-12-2	>200	>200	No	Neg	3.8 & 4.3	NS	Neg (tested up to 25%, 6250)	Neg @ 6496% (n = 99, 1:3 EtOH:DEP)	Neg @ 6900	Neg @ 100% (n = 10 test, 10 control)	Neg	Neg	Pos
Methyl dihydrojasmonate	24851-98-7	68.14-144.44	>200	No	Pos	2.79, 2.81 & 3.1	NS	Neg (tested up to 40%, 10,000)	Multiple studies; 2× Neg @ 10,000 (n = 111 and 100, 1:3 EtOH:DEP); neg @ 7752 (n = 23, SDA39C); neg @ 1876 (n = 23, SDA 39C)	Neg@13,800	multiple studies: pos and neg (positive studies disregarded due to unequivocal results); Neg @ 10% (n = 11) (Buhler)	Pos	Neg	Pos
1-(3-Methyl-2-benzofuranyl) ethanone	23911-56-0	>200	>200	No	Neg	2.52 (Calculated)	NS	Neg (tested up to 30%, 7500)	Neg @ 20% (11,019, n = 107, 1:3 EtOH:DEP)		Neg @ 50% (n = 20 test, 10 control)	Neg	Pos	Pos
2-Methyldecanenitrile	69300-15-8	<10	>200	No	Pos	4.2	NS	Neg (up to 100%, 25,000)	Neg @ 2250 (n = 101; 1:3 EtOH:DEP)		Neg @ 30% (n = 10 test, 4 control); Neg @ 100% (10 test, 10 control) (Buehler)	Neg	Neg	Pos
Ethylene brassylate	105-95-3	NA (20.45)	>200	No	Pos	4.7	NS	Neg (tested up to 30%, 7500)	Multiple studies: Neg at 11810 [6], n = 65, 91, 95, 99, 22, 109; pos @ 11,810, n = 107, 125; Neg at 23620 [4], n = 48, 71, 35, 103; Pos at 23620 [5], n = 50, 64, 67, 46, 109	Neg @ 20,700	Pos and Neg	Neg	Neg	Pos
Propanedioic acid, 1-(3,3-dimethylcyclohexyl) ethyl, ethyl ester (Trade-Musk nouvelle/ Applelide)	478695-70-4	NA (38.89)	>200	No	Neg	4.9	NS	Neg (tested up to 100%)	Neg @ 2000 (n = 107, 3:1 EtOH:DEP)			Neg	Neg	Pos
2-Isobutyl-4-methyltetrahydro-2H-pyran-4-ol	63500-71-0	189	>200	No	Pos	1.65	NS	Neg (tested up to 30%, 7500)	2 studies: Neg @4408 (n = 110, 1:3 EtOH:DEP); Neg @ 5000 (n = 50, pet)		Neg@100% (n = 20 test, 20 control); Neg @99% (n = 20 test, 10 control)	Neg	Neg	Pos
B. Sensitizers														
6-Methyl-3,5-heptadien-2-one	1604-28-0	9.31	55.09	Yes	Pos	2	110	Neg (tested up to 5%, 1250)	Neg @ 118 (n = 105, 1:3 EtOH:DEP); Pos @ 1299 (n = 3/110, 1:3 EtOH:DEP); Pos @2710 (n = 6/48, phthalate ethyl)			Pos	Pos	Pos
5,6,7-Trimethylocta-2,5-dien-4-one	358331-95-0	0.45	12.34	Yes	Pos	3.3 (Calculated)	250	Pos (EC3 = 1.6%, 400)	Neg @ 250 (n = 97, vehicle unspecified)			Inconclusive	Pos	Pos
3-Methyl-5-phenylpent-2-enenitrile	93893-89-1	14.21	>200	No	Pos	2.93 at 23.6 °C (weighted mean); 2.81 and 2.98 (isomer 1 and 2, respectively)	270	Pos (EC3 = 0.77%,192)	2 studies: Neg @275 (n = 110, 1:3 EtOH:DEP); Neg @ 27.5 (n = 107; 1:3 EtOH:DEP)			Neg	Neg	Pos
Tea Leaf Absolute	68916-73-4 / 84650-60-2	94.91	>200	Yes	Pos	NA	480	>5%, (1250)	Neg @ 480		Pos (Buehler)	Pos	Neg	Pos
1,2,3,4,5,6,7,8-Octahydro-8,8-dimethyl-2-naphthaldehyde	68991-97-9	2.96	38.1	Yes	Pos	4.51 ± 0.0042	550	Pos (EC3 = 4.5%; 1050)	Neg @ 1103 (n = 56, 3:1 EtOH:DEP); Neg @ 551 (n = 56, alcohol); Neg @551 (n = 52, 3:1 EtOH:DEP) Neg @ 5078 (n = 110, 1:3 EtOH:DEP)			Neg	Neg	Pos
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	3658-77-3	16.09	>200	No	Pos	0.82 (Calculated)	590	Pos (EC3 = 1.8%, 459)	Multiple studies: Pos @ 1181 (n = 1/110, 1:3 EtOH:DEP);			Pos	Neg	Pos

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Table 1 (continued)

		U-SENS™ RESULTS			HISTORICAL DATA									
Name	CASRN	Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% µg/cm²)	CNIH (µg/cm²)	HMT (µg/cm²)	GPMT or Buehler	DPRA	KS	H-CLAT
Eucalyptol	470-82-6	NA (52.17)	>200	No	Pos	3.4	590	Pos (EC3 = 65.9%, 16,475)	Pos @ 550 (n = 7/107, 1:3 EtOH:DEP; possible pre-sens); Neg @ 1181 (n = 105, 1:3 EtOH:DEP); Neg @591 (n = 108, 1:3 EtOH:DEP)			Neg	Pos	Pos
Methyl 2,4-dihydroxy-m-toluate	33662-58-7	32.96	>200	No	Pos	2.67 (Calculated)	620	Pos (EC3 = 8.8%, 2200 - no dose response)	Neg @ 590 (n = 108, 1:3 EtOH: DEP)	Neg @ 0.055 (n = 112, 1:3 EtOH:DEP); Neg @ 620 (n = 104, 1:3 EtOH:DEP)		Inconclusive	Pos	Pos
Allyl phenoxyacetate	7493-74-5	>200	>200	No	Neg	2.5 & 2.33	700	Pos (EC3 = 3.1%, 775)	2 studies: Neg @ 709 (n = 108, 2 studies: Neg @ 690 (n = 25 Neg @ 100% (n = 10) and 26) (Buehler)	Neg at 10% (n = 10); Neg 1:3 EtOH:DEP); neg @ 1% (n = 10)		Pos	Pos	Pos
p-Mentha-1,8-dien-7-al	2111-75-3	16.32	86.32	Yes	Pos	3.34 (Calculated)	700	2 studies: Pos (EC3 = 8.4%, 2025); (EC3 = 9.3%, 2325)	Neg @ 709 (n = 116, 1:3 EtOH:DEP)	Pos @ 2760; Neg @690		Pos	Pos	Pos
Oakmoss	9000-50-4 / 90028-68-5	10.89	58.4	Yes	Pos	NA	700	Multiple Pos (3.5–40.5)	Neg @700	Neg @ 1724		Pos	Pos	Pos
Treemoss	68648-41-9/ 90028-67-4	7.16	32.71	Yes	Pos	NA	700	Pos and Neg	Neg @930	Neg @ 6900		Pos	Pos	Pos
3-Propylidenephthalide	17369-59-4	10.08	>200	Yes	Pos	2.03 (Calculated)	940	2 studies: Pos (EC3 = 3.7%, 925); (EC3 = 1.4%, 350)	2 studies: Neg @ 945 (n = 109, 2 studies: Neg @ 193 (n @ 345; Pos @ 2760 = 38, alcohol)			Pos	Neg	Pos
Cinnamyl nitrile	1885-38-7	99.47	>200	No	Pos	1.84 (Calculated)	1000	Neg (tested up to 10%, 2500)	Multiple studies: Neg @ 1060 (n = 118, 1:3 EtOH:DEP); Pos @ 1250 (n = 54, SDA39C); Pos @ 1938 (n = 2/41, EtOH); Neg @ 969 (n = 37, SDA39C)	Neg @3450		Neg	Pos	Pos
p-Tolualdehyde	104-87-0	NA (10.95)	>200	No	Pos	1.9	1000	Pos (EC3 = 0.69%, 172.5)			Neg @100% (n = 10 Neg test, 5 control)		Neg	Pos
7-Methyl-2H-benzo-1,5-dioxepin-3 (4H)-one	28940-11-6	37.95	>200	Yes	Pos	1.95	1000	Neg (tested up to 30%, 7500)	Neg @ 1000 (n = 97, unknown vehicle)			Pos	Pos	Pos
p-tert-Butyldihydrocinnamaldehyde	18127-01-0	10.33	18.46	Yes	Pos	3.2	1100	Pos (EC3 = 4.3%, 1075)	Multiple studies: Neg @ 1181 (n = 77, 24, and 30, 1:3 EtOH: DEP); Neg @ 7000 (n = 26; 1:3 EtOH:DEP)	Neg @ 4140	2 studies: Pos @ 50% pos (n = 10 test, 4 control); Pos @ 25% (n = 10 test, 5 control); Pos @25% (n = 10 test, 5 control) (Buehler)		neg	pos
Cuminaldehyde	122-03-2	>200	>200	No	Neg	2.8	1100	Neg (tested up to 10%, 2500)	Neg @ 1181 (n = 105, 1:3 EtOH:DEP)	Neg @ 2760	Pos (limited details)	Pos	Neg	Borderline
3-(p-Isopropylphenyl) propionaldehyde	7775-00-0	5.95	26.32	Yes	Pos	3.49 (Calculated)	1100	Pos (EC3 = 18.6%, 4650)	Neg @ 1111 (n = 99, 3:1 SD39C:DEP)			Neg	Neg	Pos
Jasmine Absolute (Grandiflorum)	8022-96-6 / 84776-64-7	5.58	120	Yes	Pos	NA	1400	EC3 = 5.9% (1475)	Neg @17,475	Multiple studies: Pos and Neg @ 2070		Pos	Pos	Pos
ylang ylang	8006-81-3/ 68606-83-7/ 83863-30-3	94.38	>200	Yes	Pos	NA	1700	EC3 > 10, EC3–6.8 (1700)	Neg@1771	Neg @ 6900		Neg	Pos	Pos

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Table 1 (*continued*)

Name	CASRN	U-SENS TM RESULTS			HISTORICAL DATA								
		Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% μ g/cm ²)	CNIH (μ g/cm ²)	HMT (μ g/cm ²)	GPMT or Buehler	DPRA	KS H-CLAT
Dibenzyl ether	103-50-4	>200	>200	No	Neg	3.4	2300	Pos (EC3 = 6.3%, 1575)	Neg @ 2362 (n = 108, 1:3 EtOH:DEP)	Neg @2760	Neg @ 5% (n = 20 test, 10 control)	Neg	Pos Pos
p-Isobutyl- α -methyl hydrocinnamaldehyde	6658-48-6	4.56	142.11	Yes	Pos	3.8	2300	Pos (estimated EC3 = 9.5%, 2375)	Neg @ 2362 (n = 104, 1:3 EtOH:DEP)		Pos @ 2% (n = 5 test, Neg 5 control)		Neg Pos
3,3-Dimethyl-5-(2,2,3-trimethyl-3-cyclopenten-1-yl)-4-penten-2-ol	107898-54-4	NA (20.23)	43.92	Yes	Pos	5.26, 4.33, 4.989 & 4.91	2500	Neg (tested up to 20%, 5000)	Multiple studies: Neg @ 2598 (n = 104, 1:3 EtOH:DEP); Pos @5000 (n = 109; DEP); Neg @ 10,000 (n = 107, 1:3 EtOH: DEP)		Neg @ 100 (n = 20 test, 20 control)	Pos	Neg Pos
β ,4-Dimethylcyclohex-3-ene-1-propan-1-al	6784-13-0	43.58	>200	No	Pos	4.1	2500	3 studies: Pos (EC3 = 8.12%,2030) (EC3 = 37.4%, 9350); Neg (tested up to 50%, 12,500)	Multiple studies: neg @ 2598 (with toco, n = 110, 1:3 EtOH: DEP); Pos @ 5510 (with toco, n = 3/102, 1:3 EtOH:DEP); Neg @ 5510 (with BHT, n = 105, 1:3 EtOH:DEP)		Pos @25% (n = 10 test, 10 control) (Buehler)	Neg	Pos Pos
Methoxy dicyclopentadiene carboxaldehyde	86803-90-9	20.29	NA (47.63)	Yes	Pos	2.63 (Calculated)	2500	>2500	2 studies: Neg up to 25% (n < 40)		Pos @ 50%	Pos	Pos Pos
1-(1-Naphthyl)ethanone	941-98-0	>200	>200	No	Neg	2.85 (Calculated)	2500	Pos (EC3 = 10%, 2500)	Neg @ 2598 (n = 108, 1:3 EtOH:DEP)	Neg @ 1380		Inconclusive	Neg Pos
1-(5,5-Dimethyl-1-cyclohexen-1-yl)pent-4-en-1-one	56973-85-4	NA (22.42)	>200	No	Pos	3.64 & 4.1	2500	Pos (EC3 = 3%, 750)	Multiple studies: Neg @ 2500 (n = 105, 3:1 EtOH:DEP); Neg @ 500 (n = 102, DEP); Neg @2500 (n = 100, DEP); other insuff Neg studies		Pos @ 100% (n = 10); Pos @75% (n = 15);	Neg	Pos Pos
Heptaldehyde, ethylene glycol acetal	1708-34-5	NA (43.54)	>200	No	Pos	2.57 (Calculated)	2700	Pos (EC3 = 64.98%, 16,245)	Neg @ 2777 (n = 106, 1:3 EtOH:DEP)			Neg	Neg No data
2-Methyl-4-(2,6,6-trimethylcyclohex-1-en-1-yl)-2-butenal	3155-71-3	6.2	20.76	No	Pos	5.24 (Calculated)	2900	2 studies: Pos (EC3 = 11.9%, 2975); Pos at all conc. (EC3 < 1%, <250)	Multiple studies: Neg @ 2953 (n = 107, 1:3 EtOH:DEP); Pos @ 10,526 (n = 1/20, 1:1 EtOH: DEP); Neg @ 968 (n = 41; ethanol)		2 studies: Pos @ 50% Pos (n = 20 test, 20 control); Pos @ 100% (n = 20 test, 16 control);		Neg Pos
Piperonal	120-57-0	>200	>200	No	Neg	1.05 & 1.2	2900	Pos (EC3 = 25%, 6250)	Neg @ 2953 (n = 112, 1:3 EtOH:DEP)	Neg @4140	Neg @ 10% (n = 5)	Pos	Pos Pos
Butanamide, 2-ethyl-N-methyl-N-(3-methylphenyl)-	406488-30-0	0.18	166	Yes	Pos	3.2	3200	Pos (EC3 = 25%, 6250)	Neg @ 3250 (n = 101, 1:3 EtOH:DEP)			Neg	Neg Pos
1-Octen-3-yl acetate	2442-10-6	>200	>200	No	Neg	3.47	3500	Neg (tested up to 30%, 7500)	2 studies: Neg @ 3543 (n = 109, 1:3 EtOH:DEP); Neg @968 (n = 38, unknown veh) 6900	7 studies: Pos and Neg @ 6900	Neg Modified Buehler (n = 20 test, 10 control)	Neg	Pos Neg
p-Methoxybenzaldehyde	123-11-5	NA (139.4)	>200	No	Pos	1.79 (Calculated)	3500	Neg (tested up to 25%, 6250)	Multiple studies: Neg @ 3543 (n = 102, 1:3 EtOH:DEP); Neg @ 2363 (n = 109; 1:3 EtOH: DEP); Pos @ 4724 (n = 1/111, 1:3 EtOH:DEP); Pos @ 6495 (n = 1/109; 1:3 EtOH:DEP)	Neg @ 6900		Pos	Neg Pos
Formaldehyde cyclododecyl ethyl acetal	58567-11-6	46.96	>200	No	Pos	5.4 & > 6.0	3500	Pos (EC3 = 25.1%, 6275)	Neg @ 3543 (n = 112, 1:3 EtOH:DEP)	Neg @ 1380		Neg	Neg Pos
Vanillyl butyl ether	82654-98-6	NA (1.79)	>200	Yes	Pos	2.2	3500	Pos (EC3 = 14.6%, 3650)	Neg @ 3543 (n = 104, 1:3 EthOH: DEP)		2 studies: Pos @ 100% (10 test and 5 control); Pos @ 10%	Pos	Pos Pos

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Table 1 (*continued*)

Name	CASRN	U-SENS™ RESULTS			HISTORICAL DATA									
		Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% µg/cm ²)	CNIH (µg/cm ²)	HMT (µg/cm ²)	GPMT or Buehler	DPRA	KS	H-CLAT
Longifolene	475-20-7	>200	>200	No	Neg	>6.0	3500	Pos (EC3 = 1.75%, 437); (EC3 = 9.4%, 2350); (EC3 EtOH:DEP) = 31.4%, 7850)	Neg @ 3543 (n = 105, 1:3	Neg @ 6900	(n = 5 test and 5 control)	Neg	Pos	Pos
β-Farnesene	18794-84-8	4	NA (21.16)	Yes	Pos	7.17 (Calculated)	3700	2 studies: both Neg (tested up to 30% and 25%, 7500 and 6250)	3 studies: Neg @ 3779 (n = 110; 1:3 EtOH:DEP); Pos @6350 (1/51, alcohol); Neg @ 3750% (n = 51, alcohol)		Pos @ 0.1% and 1.0% (n = 15 test, 4 control)	neg	pos	Pos
2,4,6-Trimethyl-3-cyclohexene-1-methanol	68527-77-5	43.93	>200	Yes	Pos	3.3 (Calculated)	3800	Neg (tested up to 25%, 6250)	Neg @ 3897 (n > 100), pos @ 7751 (n = 2/49, SDA39C); Neg @ 3875 (n = 51, SDA37C)		3 Buehler studies: Pos @ 1%, 4%, and 7.5% (n = 10 in each study) Pos @ 60% (7/10) (Buehler)	Neg	Neg	Pos
omega-Pentadecalactone	106-02-5	16.78	>200	No	Pos	5.78 & > 6	5500	Multiple studies: Pos (EC3 = 25%, 6250); (EC3 = 16.6%, 4150); (EC3 < 10%, 2500)-these may be due to impurities; Neg (purified sample, tested up to 50%, 12,500)	Neg @ 5500 (n = 105, 1:3 EtOH:DEP)	Neg @ 6900	2 studies: Pos and Neg @100 (n = 20 test nd 10 control each study)	Neg	Neg	Pos
trans-Anethole	4180-23-8	>200	>200	No	Neg	3.39 (Calculated)	5500	Pos (EC3 = 2.7%, 675)	Neg @ 5500 (n = 105, 1:3 EtOH:DEP); Neg @ 2254 (n = 101, 1:3 EtOH:DEP)	Neg @1380		Inconclusive	Neg	Pos
Benzyl alcohol	100-51-6	>200	>200	No	Neg	1.1	5900	Neg (tested up to 50%, 12,500)	5 studies: Neg @3543 (n = 101, 1:3 EtOH:DEP); Pos @ 8858 (n = 1/110, 1:3 EtOH:DEP); Neg @ 3543 (n = 107, 1:3 EtOH:DEP); Neg @ 23,620 (n = 56, 1:3 EtOH:DEP), Neg @17,715 (n = 46, 1:3 EtOH:DEP)	Neg @ 6900 (n = 24)		Neg	Neg	Pos
4-Methoxy-alpha-methylbenzenepropanal	5462-06-6	5.49	108.33	Yes	Pos	1.74 & 2.5	5900	Pos (EC3 = 23.6%, 5900)	4 studies: Neg @ 5906 (n = 104; 1:3 EtOH:DEP); Neg @1250 (n = 53; SDA39C); Neg @ 1000 (n = 50; unknown vehicle); Neg @ 387 (n = 38, alcohol);	Neg @ 1380	2 studies: Pos @ 100% (n = 10 test 5 control); Neg @ 5% (n = 10 test)	Neg	Pos	Pos
3-Phenylbutanal	16251-77-7	3.14	8.97	Yes	Pos	1.9	5900	None	Multiple studies: Neg @ 5906 (n = 102, 1:3 EtOH:DEP); Pos @ 12,500 (n = 3/47, alcohol); Neg @ 2500 (n = 100, DEP)		Neg@100% (n = 10 test, 10 control) (Buehler)	Pos	Pos	Pos
3-(4-Methyl-3-cyclohexenyl) butanol	15760-18-6	30.75	>200	Yes	Pos	3.3 & 3.79	5900	Pos (EC3 = 20%, 5000)	Neg @ 5906 (n = 107, DEP); Neg @ 12,500 (n = 47, anhydrous alcohol)			Neg	Pos	Pos
2-Methyldecanal	19009-56-4	NA (1.15)	<10	Yes	Pos	4.5	5900	Pos (EC3 = 23.6%, 5900)	Neg @ 5905 (n = 102; 1:3 EtOH:DEP); Neg @ 388 (n = 39)	Neg @ 6900		Pos	neg	pos
2,4-Dimethyl-3-cyclohexen-1-carboxaldehyde	68039-49-6	7.66	34.42	Yes	Pos	2.7, 2.8, & 3.1	5900	Multiple studies: 5× Pos (EC3 = 25.14%, 6285); (EC3 = 10.5%, 2625);	Multiple studies: Neg@ 5905 (n = 108, 1:3 EtOH:DEP); other insufficient Neg studies		Multiple studies: Pos and Neg;	Pos	Pos	Pos

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Table 1 (continued)

Name	CASRN	U-SENS™ RESULTS			HISTORICAL DATA								
		Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% μ g/cm ²)	CNIH (μ g/cm ²)	HMT (μ g/cm ²)	GPMT or Buehler	DPRA	KS H-CLAT
6-Methoxy-2,6-dimethylheptan-1-al	62439-41-2	NA (13.21)	NA (>30)	Yes	Pos	2	5901	(EC3 = 20%, 5000); (EC3 = 2.34%, 585); (EC3 = 5.38%, 1345); Neg (tested up to 25%, 62,500) 2 studies: Pos (EC3 = 24%, 6000); Neg (tested up to 50%, 12,500)	Neg @ 5905 (n = 106, 1:3 EtOH:DEP)		Pos and Neg (Buehler)	Pos	Pos pos
Isocyclocitral	1335-66-6	85	>200	No	Pos	3.1	7000	Pos (EC3 = 7.3%, 1825)	2 studies Neg @ 7078 (n = 77 Neg @ 2760 and 28, 1:3 EtOH:DEP); another study Neg @ 96 (n = 37, ethanol)	4 studies: 2× Pos@ 50% (n = 10 test, 4 control); 2× Pos@25% (n = 10 test, 4 control)	Pos	Neg	Neg Pos
Citronellal	106-23-0	NA (1.67)	NA (88.51)	Yes	Pos	3.53 (Calculated)	7000	Neg (tested up to 30%, 7500)	Neg @ 7086 (n = 110, 1:3 EtOH:DEP)	Neg @ 2760	Pos (n = 8 test) Pos @10% (n = 10 test) (Buehler)	Neg	Pos Pos
Jasmine Absolute (Sambac)	91770-14-8 / 1034798-23-6	13.71	>200	Yes	Pos	NA	8800	EC3 = 36.4% (9100)	Neg @8858			Neg	Pos Pos
beta,beta,3-Trimethyl benzenepropanol	103694-68-4	44.77	133.33	Yes	Pos	3.07	9900	4 studies: Pos (EC3 29.2%, 7300), Neg (tested up to 30%), Neg (tested up to 100%), Neg (tested up to 100%)	2 studies: Neg @9917 (n = 98, 1:3 EtOH:DEP); Neg @ 5509 (n = 103, 1:3 EtOH:DEP)		2 studies: Neg @ 1% and 5% (n = 20 test, 20 control)	Neg	Pos Pos
3-Methylcyclopentadecenone	82356-51-2	9.42	34.81	Yes	Pos	>4.88	10,000	1425 (EC3 = 5.7%); pos	Neg @ 10,000 (DEP); Neg @ 5000		Neg @ 100% (n = 20); Neg @ 50% (n = 20) (Buehler)	No data	Neg Pos
5-Cyclotetradecen-1-one, 3-methyl-, (5E)-	259854-70-1	<1	>10	Yes	Pos	5.6	10,000	Pos (EC3 = 16.4%, 4100)	Multiple studies: Neg @ 5000 (n = 103, 1:3 EtOH:DEP); Neg @ 10,000 (n = 97, 1:3 EtOH:DEP); Neg @ 3000 (n = 54, DMP)			Neg	Neg Pos
1,1,3-Trimethyl-3-phenylindane	3910-35-8	NA (7.36)	13.88	Yes	Pos	5.91 (Calculated)	10,000	Pos (EC3 = 43.3%, 10,825)	Neg @ 10,630 (n = 106, 1:3 EtOH:DEP)			Neg	Pos Inconclusive
alpha-Methyl-1,3-benzodioxole-5-propionaldehyde	1205-17-0	8.83	41.32	Yes	Pos	2.4	11,000	Pos (EC3 = 16.4%, 4100)	Multiple studies: Neg @ 11,811 (n = 109, 1:3 EtOH:DEP); Neg @ 1581 (n = 115, 1:3 EtOH:DEP); Neg @ 10,000 (n = 51, SD39C); Neg @ 10,000 (n = 48, 3:1 EtOH:DEP); Pos @ 30% (n = 107, 3:1 EtOH:DEP)	Neg @ 13,800		Pos	Pos Pos
Tetrahydro-4-methyl-2-propyl-2H-pyran-4-yl acetate	131766-73-9	46.57	>200	No	Pos	2.39	11,000	Neg (tested up to 30%, 7500)	Multiple studies: Neg up to 11,019 (n = 106; 1:3 EtOH:DEP); Neg @8264 (n = 50, 3:1 SD39C:DEP); Neg @5510 (n = 55, 3:1 SD39C:DEP); Other Neg studies in alcohol		Pos @ 100 (n = 20 test, 10 control); Neg @ 15% (n = 20 test, 10 control) (Buehler)	Neg	Neg Pos
Methyl atrarate	4707-47-5	>200	>200	No	Neg	2.1	11,000	2 studies: Pos (EC3 = 19%, 4750); Neg (tested up to 25%, 6250)	Multiple studies: Neg @ 10% (11,810, n = 100, 1:3 EtOH:DEP); Neg @ 5% (2500, n = 54, no vehicle); Neg @ 2.5% (1938, n = 44, SDA 39C)	Neg @ 6900	Neg @ 25% (limited info)	inconclusive	Pos Pos

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Table 1 (continued)

		U-SENS™ RESULTS				HISTORICAL DATA								
Name	CASRN	Mean EC150	Mean CV70	Interference	Prediction	Log Kow	NESIL	LLNA (% µg/cm²)	CNIH (µg/cm²)	HMT (µg/cm²)	GPMT or Buehler	DPRA	KS	H-CLAT
cis-4-(Isopropyl) cyclohexanemethanol	13828-37-0	28.18	>200	No	Pos	3.48		17,000 Pos (EC3 = 44%, 11,000)	Multiple studies: Neg @17,717 (n = 100, 1:3 EtOH:DEP); Other studies Neg in diff. Vehicles, at lower doses			Neg		Neg Pos
2-Nonyn-1-al dimethyl acetal	13257-44-8	>200	>200	No	Neg	3.6		23,000 Neg (tested up to 20%, 5000)	Neg @ 23,622 (n = 108, 1:3 EtOH:DEP)	Neg@2760	Pos@ 20% (n = 10 test, 4 control)	Neg	Pos	Pos
1-(1,2,3,4,5,6,7,8-Octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone	54464-57-2	NA (21.34)	21.34	Yes	Pos	5.6 & 5.7 for the 2 isomers	47,000	3 studies: Pos (EC3 = 25.14%, 6285) (EC3 = 6.07%, 1518) (EC3 = 14.2%, 3550)	7 studies: Neg @ 47,244 (n = 101, 1:3 EtOH:DEP); 2× Neg @ 1938 (n = 36 and 42, SDA39C); Neg @ 1550 (n = 44, SDA39C); Neg @6250 (n = 51, SDA 39C); Neg @9297 (n = 53, 3:1 acohol:DEP); neg @7231 (n = 52, 3:1 alcohol: DEP)			Pos		Neg Pos

Pos: Positive.

Neg: Negative.

EC3: Concentration of test chemical required to induce a 3-fold increase in lymph node cell proliferation.

EC150: Concentration at which a chemical reaches the CD86 positive threshold of 150%.

CV70: Concentration showing 70% cell viability.

LLNA: Local lymph node assay.

CNIH: Confirmation of no induction in humans.

HMT: Human maximization test.

GPMT: Guinea pig maximization tests.

DPRA: Direct peptide reactivity assay.

KS: KeratinoSensTM.

H-CLAT: human cell line activation test.

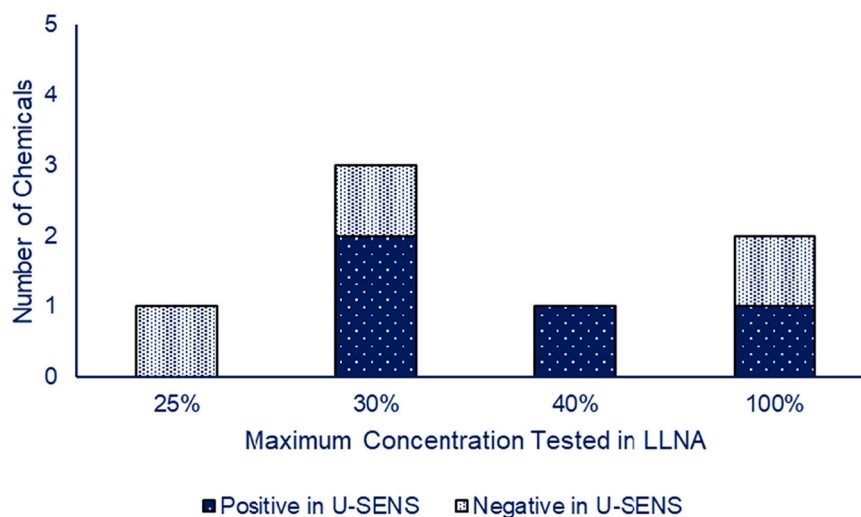


Fig. 1. The 7 non-sensitizer fragrance ingredients had been tested in the LLNA at concentrations ranging from 25% to 100%. Of the 7 non-sensitizers, 3 were predicted to be negative by U-SENS™ while 4 were predicted to be positive. The darker shaded bars were positive in U-SENS™, while the lighter shaded ones were negative.

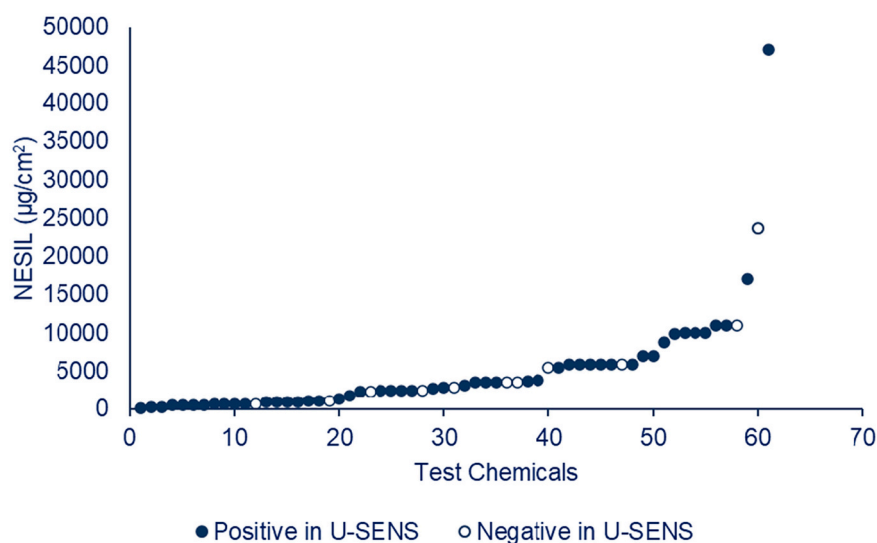


Fig. 2. 50 sensitizers were predicted positive by U-SENS™ while the remaining 11 were negative. Each circle represents a chemical. The open circles are the chemicals that gave negative results from U-SENS™, while the filled in circles were positive in U-SENS™.

predicted sensitization potential of fragrance ingredients with a high PPV of 93% when compared to WoE from all historical data, but the NPV was 21%, suggesting that U-SENS™ may predict fragrance ingredient sensitizers with greater confidence than non-sensitizers, even though the non-sensitizer dataset was too small to make definitive conclusions. An additional analysis was done comparing U-SENS™ to just the LLNA and the PPV dropped to 70%, while the NPV rose to 50%. These data show that U-SENS™ predicts sensitizers with higher confidence when compared to WoE from all combined historical data (human, LLNA, guinea pig maximization, and Buehler) as opposed to just the LLNA. While measuring U-SENS™ against the LLNA improves the NPV, for the purposes of protection of humans against skin sensitization it is better to be conservative in predicting sensitizers with higher confidence than predicting non-sensitizers. Results from this dataset of 68 fragrance ingredients showed that the U-SENS™ assay offers a wide applicability domain, as none of the physical-chemical properties evaluated, such as solubility and interference, were shown to influence how well the assay predicted sensitizers *versus* non-sensitizers.

In silico prediction of protein binding alerts can provide some insight into the ability of a chemical to be sensitizing. There are 6 essential protein binding mechanisms that act directly to initiate skin sensitization, including first-order nucleophilic substitution (SN1), second-order nucleophilic substitution (SN2), nucleophilic substitution aromatic (SNAr), acylation (A), Schiff-base formation (SB), and Michael-acceptor (MA). Only 3 of these mechanisms (A, SB, and MA) are of significance in explaining skin sensitization by fragrance ingredients. Protein binding alerts were predicted using the OECD Toolbox 4.3 and Toxtree 3.1.0 to provide insight into the reactivity of the parent chemicals and their respective metabolites. Protein binding alerts for NCS were not determined, as these chemicals comprise several discrete fragrance ingredient components. Within the chemical set that was determined to be positive in U-SENS™, 39% did not have direct parent protein binding alerts. However, most of these chemicals had a metabolite protein binding alert, suggesting that the parent chemicals may have been prohaptens metabolized to the protein binding haptens.

U-SENS™, like other *in vitro* skin sensitization assays based on the

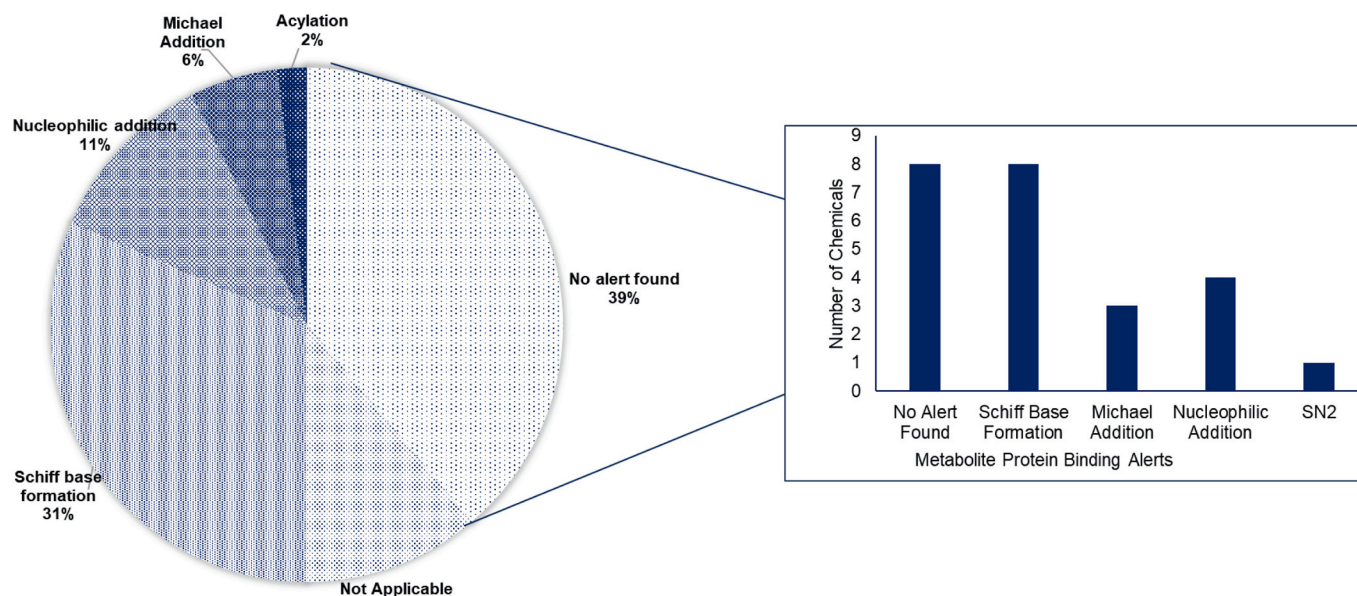


Fig. 3. Protein binding alerts of fragrance ingredients predicted to be positive in U-SENSTM. Pie chart: 27 of the 47 discrete chemicals predicted to be positive in U-SENSTM had parent protein binding alerts. Bar graph: 13 of the test chemicals that were positive in U-SENSTM, with no parent alert generated metabolite protein binding alerts.

		U-SENS™		
		Negative	Positive	
DPRA	Negative	9	28	52% Match
	Positive	2	23	
Keratinosens	Negative	6	28	47% Match
	Positive	8	26	
h-CLAT	Negative	1	0	82% Match
	Positive	12	52	

Fig. 4. Concordance between U-SENSTM and other *in vitro* assay predictions in the skin sensitization AOP.

Table 2

Positive and negative predictive values: U-SENSTM compared to *in vivo* and *in vitro* data.

U-SENS TM comparison with	Positive predictive value (PPV, %)	Negative predictive value (NPV, %)
WoE from all <i>in vivo</i> data	91	21
LLNA Only	70	50
2 out of 3 call (DPRA, KeratinoSens TM and U-SENS TM)	97	24

AOP, was not developed as a standalone assay. The “2 out of 3” defined approach is one of several developed combining some *in vitro* assays to assess the skin sensitization potential of chemicals (Kolle et al., 2019; OECD, 2020a). Specifically, the “2 out of 3” DA combines the DPRA, KeratinoSensTM, and h-CLAT, and a chemical is determined to be a sensitizer or non-sensitizer based on 2 out of 3 positive or negative, respectively, predictions out of the 3 *in vitro* assays. In addition to historical *in vivo* data, most of the fragrance ingredients in the tested set also had conclusive results from the DPRA, KeratinoSensTM, and h-CLAT,

addressing key events 1, 2, and 3, respectively. Within the chemical set with concordance between DPRA and KeratinoSensTM results (32 chemicals), PPV and NPV of 100% and 26% was observed. Combining U-SENSTM with the DPRA and KeratinoSensTM in a “2 out of 3” call yielded PPV and NPV of 97% and 23%, respectively, when measured against WoE from historical *in vivo* data. These results confirm that U-SENSTM predicts sensitizers with higher confidence when used in the “2 out of 3” defined approach. Additionally, adding U-SENSTM to the DPRA and KeratinoSensTM dataset increased the number of chemicals for which definitive conclusions could be made to 64 without significantly changing the PPV and NPV. A similar analysis replacing U-SENSTM with h-CLAT yielded comparable results with PPV and NPV of 95% and 25%, respectively, thus confirming the similarity between these two assays addressing dendritic cell activation. Further analysis showed that U-SENSTM and h-CLAT had an 82% concordance, while lower concordance of 52% and 47% were observed when U-SENSTM was compared to the DPRA and KeratinoSensTM, respectively. This analysis demonstrates that combining *in vitro* studies with higher dissimilarity (as was demonstrated by combining DPRA, KeratinoSensTM, and U-SENSTM) may be more beneficial in predicting skin sensitization hazard than those addressing the same key event. Therefore, either U-SENSTM or h-CLAT may be combined with the DPRA and KeratinoSensTM in skin sensitization defined approaches.

The OECD guideline on defined approaches for skin sensitization (guideline number 497) uses non-animal data to predict skin sensitization hazard and potency (OECD, 2021). These defined approaches amalgamate data from different sources, including the DPRA, KeratinoSens assay, h-CLAT, in combination with read-across predications and physico-chemico properties in the prediction of skin sensitizers. Given the similarity in applicability domain between the h-CLAT and U-SENSTM, U-SENSTM may be interchangeable with the h-CLAT in these defined approaches. While h-CLAT has testing limitations for chemicals with log Kow > 3.5 (tendency for false negative results) (OECD, 2018b), U-SENSTM has been demonstrated to correctly classified the substances with a log Kow > 3.5 (Alépée et al., 2017). Therefore, U-SENSTM may be a good key event 3 *in vitro* assay for test chemicals with high log Kow within the defined approaches.

Currently, U-SENSTM is not recommended as a standalone assay and must be used in combination with other *in vitro* assays to predict hazard.

However, this assay has potential for further development to provide insight into chemical potency. U-SENS™ EC150 is a threshold chemical concentration at which 1.5-fold induction of the CD86 cell surface marker is observed. It may be hypothesized that more potent or stronger skin sensitizers have the potential to activate CD86 1.5-fold at lower concentrations than weaker sensitizers. Therefore, further investigations may reveal the U-SENS™ EC150 as a tool to predict sensitizer potency.

In conclusion, we demonstrate the ability of the U-SENS™ assay to predict the sensitization potential of fragrance ingredients and recommend combining U-SENS™ with key event 1 and 2 *in vitro* assays for hazard identification in the safety assessment of fragrance ingredients. *In silico* analysis demonstrate that metabolism may explain the positive results obtained for chemicals without direct protein binding alerts. No physico-chemico properties evaluated could explain the disparities between U-SENS™ results and conclusions from WoE assessment of historical data, suggesting that U-SENS™ has a wide applicability domain within fragrance ingredients.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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