

ORIGINAL ARTICLE

Common fragrance chemicals activate dendritic cells in coculture with keratinocytes

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Abstract

Background: Fragrances are important contact allergens; however, investigation of their skin sensitization potency has been challenging in new approach methods (NAMs). Many fragrance chemicals are susceptible to autoxidation or can be metabolized by enzymes constitutively expressed in skin keratinocytes. Strong sensitizers can be formed in both of these processes. Further, keratinocytes can modulate the dendritic cell (DC) activation and maturation potential, a key event in the acquisition of contact allergy.

Objectives: To evaluate the 2D coculture model consisting of keratinocytes and DCs using different weak to moderate sensitizing fragrance chemicals. Further, to investigate fragrances and related oxidation products in the in vitro model and compare to in vivo data.

Methods: Chemicals were tested in the coculture activation test (COCAT), consisting of HaCaT keratinocytes and THP-1 cells. THP-1 cell surface expression of costimulatory and adhesion molecules (CD86 and CD54) collected after 24 h incubation with the chemicals was analysed using flow cytometry.

Results: Twenty-four molecules were tested positive, three were negative ($n = 27$). Four pairs were evaluated, with aldehydes showing a 6- to 13-fold stronger responses compared to their corresponding alcohols.

Conclusions: Results provide insight into the activation of DC in their natural environment of keratinocytes. α,β -Unsaturated alcohols were classified as weaker sensitizers compared to their corresponding aldehydes. In sum, testing of fragrances retrieved results in good agreement with in vivo data.

KEYWORDS

allergic contact dermatitis, cosmetics, fragrances

1 | INTRODUCTION

Skin contact to low molecular weight chemicals, especially fragrances, are frequent causes of skin sensitization and contact allergy.¹ They

rank second after nickel as the most common cause of contact allergy in the general European population.^{2,3} Upon dermal uptake the sensitizer needs to generate antigens and sufficient danger signals for the activation and maturation of dendritic cells (DC), which are key events

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in the skin sensitization process. Skin sensitization involves Langerhans cells and dermal DCs that integrate signals from their cellular environment and migrate to nearby tissue-draining lymph nodes, where they instruct naïve T cells to proliferate. DC are also involved in their differentiation into appropriate effector and regulatory T cells.^{4,5} Despite the high prevalence of contact allergy, fragrances' cellular modes of action is not well understood.^{6,7} Fragrances are predominantly classified as moderate or weak sensitizers^{3,8} and noticed as challenging test substances in murine studies when tested one by one.⁹

Fragrance chemicals comprise many different chemical structures. Examples are terpenes, alcohols, aldehydes, ketones and esters. Many of them are not allergenic themselves but their oxidation products, either outside of the body through autooxidation or mediated by enzymes inside the body, can yield potent allergens. Autooxidation is well-known for several fragrance terpenes and can produce highly sensitizing hydroperoxides.¹⁰ Upon skin contact, fragrance chemicals such as alcohols can be transformed by alcohol-, and aldehyde dehydrogenases or esterases to form sensitizing aldehydes. Corresponding enzymes are constitutively expressed in the stratified keratinocyte layers (for a review see reference 11). For hydroperoxides of the fragrance terpenes linalool, citronellol and limonene, we have previously shown that primary human keratinocytes differently modulate DC activation potentials.¹² However, DC activation of the varying fragrance structures in the natural environment of keratinocytes has not yet been studied. The coculture in vitro method presented here (coculture activation test, COCAT) is composed of HaCaT keratinocytes and monocytic THP-1 cells, a surrogate for DC.^{13–16} Thus, COCAT combines two cell types and estimates DC activation in the environment of keratinocytes.

Based on this background, we aimed to evaluate a set of fragrance chemicals and related chemicals in our developed in vitro coculture method, as fragrances can pose a challenge in in vitro methods due to their generally weak to moderate skin sensitizing properties, high volatility and hydrophobic properties.^{17,18} The test set covered typical chemical classes of fragrance materials and covered most of the 26 fragrance chemicals requiring labelling in the European Union.

Further, fragrances identified as sensitizers in clinical studies but frequently not identified or with inconsistent results in new approach methods (NAMs) such as benzaldehyde, coumarin, eugenol and isoeugenol were included.¹⁹ Chemical structures known to be transformed enzymatically by keratinocytes like cinnamyl alcohol and cinnamaldehyde were also integrated.^{20–22} Furthermore, oxidation products from autooxidation of the weak sensitizer geraniol namely geranial, neral and geraniol-6/7-hydroperoxides²³ were included in order to perform a side-by-side comparison.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Chemicals were obtained from Sigma Aldrich, Taufkirchen or as indicated. Anisyl alcohol (CAS no. 105-13-5; >98%), benzaldehyde (CAS

no. 100-52-7; 99.8%), benzyl alcohol (CAS no. 100-51-6; 100%), benzyl cinnamate (CAS no. 103-41-3; 99%), (R)-(–)-carvone (CAS no. 6485-40-1; 98%), cinnamaldehyde (CAS no. 104-55-2; >95%), cinnamyl alcohol (CAS no. 104-54-1; 98%), citral (CAS no. 5392-40-5; >98%), citronellol (CAS no. 106-22-9; >99%), coumarin (CAS no. 91-64-5, >99%); dimethylsulfoxide (DMSO; CAS no. 67-68-5, >99.9%); eugenol (CAS no. 97-53-0, >98.5%); geranial (CAS no. 141-27-5, obtained from Bedoukian Research, Inc.; after purification 100%), geraniol (CAS no. 106-24-1; >97%), geraniol-6/7-hydroperoxide (CAS no. 85867-61-4/85867-60-3) was synthesized as described,²⁴ *trans*-2-hexenal (CAS no. 6728-26-3; 98%), hydroxycitronellal (CAS no. 107-75-5; 99.8%), and hydroxycitronellol (CAS no. 107-74-4; 99.5%, the latter two kindly provided by Symrise AG), 4-(4-hydroxy-4-methylpentyl)cyclohex-3-ene carbaldehyde (HICC; CAS no. 31906-04-4; >97%), isoeugenol (CAS no. 97-54-1; 99%), α -isomethylionone (CAS no. 127-51-5; >90%), limonene (CAS no. 5989-27-5; 100%), linalool (CAS no. 78-70-6; 97%), 2-methoxy-4-methylphenol (CAS no. 93-51-6; >98%), methyl-2-octynoate (methyl heptene carbonate; CAS no. 111-12-6; 99%), neral (CAS no. 106-26-3, obtained from Bedoukian Research, Inc.; after purification 96%), 1-octen-3-yl acetate (CAS no. 2442-10-6; 95%; obtained from Ambeed, Inc.), and vanillin (CAS no. 121-33-5; 99%). Materials were stored in amber HPLC vials under argon atmosphere.

Purity analysis of commercial citral found oxidation products (e.g., 6,7-epoxy-geranial) in one of the two batches, while the other batch was 100% pure.²⁴ For purification, geranial and neral were distilled under reduced pressure (20 mbar). Neral was obtained at a boiling temperature of 109–110°C and geranial at 116–117°C, respectively. For both materials, colourless liquids with a yield of 90% of the initial amount were obtained. ¹H-NMR and ¹³C-NMR confirmed the structures, with pure geranial, but neral contained about 3%–4% of geranial. Geraniol-6/7-hydroperoxide (3:5 mixture of 6-hydroperoxy-3,7-dimethyl-octa-2,7-dien-1-ol [CAS 85867-61-4] and 7-hydroperoxy-3,7-dimethylocta-2,5-dien-1-ol [CAS 85867-60-3]) was stored at –20°C under inert gas. Purity of geraniol-6/7-hydroperoxide was tested by NMR before the testing. No degradation products were detected and confirmed previous NMR signals.²³

2.2 | The COCAT

Testing was performed as previously described.¹⁶ Briefly, HaCaT cells were seeded in 96-well plates and THP-1 cells were placed on top of the HaCaT cells. The maximal test concentration for each chemical is determined based on solubility in the culture medium and/or cell viability. Cocultured cells were exposed to maximal 10 concentrations of the chemicals (1:2 dilutions, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078 mM) dissolved in final concentration of 0.2% DMSO. In each experimental run, 0.020 mM of 2,4-dinitrochlorobenzene and 0.144 mM of sodium dodecylsulfate were used as positive and negative controls, respectively. After 24 h of treatment, floating THP-1 cells were harvested and cell surface expression (mean fluorescence intensities, MFI) of CD86 and CD54 was analysed on viable (propidium iodide negative)

THP-1 cells via flow cytometry (FACSVerse™ or FACSCalibur™, BD Biosciences). Then, the chemical-induced differences in cell surface levels of CD86 or CD54 (Δ MFI) on THP-1 were calculated for each sample. Chemicals are considered as sensitizers if in ≥ 2 out of 3 runs at least one marker exceeds the threshold for positivity (Δ MFI ≥ 10.8 for CD86 or Δ MFI ≥ 300 for CD54). These thresholds were established by analysing a set of 21 sensitizers and 12 non-sensitizers in the course of the method development.^{13,14} For each chemical, the lowest positive concentration (CD86 and/or CD54) is calculated by linear interpolation or log-linear extrapolation in the case that the Δ MFI value at the lowest tested concentration already exceeded the threshold for positivity. The lowest positive concentration (either CD86 or CD54) of each run is named effective concentration (EC1-3). The mean of these values is taken as an indicator of the chemicals' potency. Figure 1 provides an overview of the experimental flow.

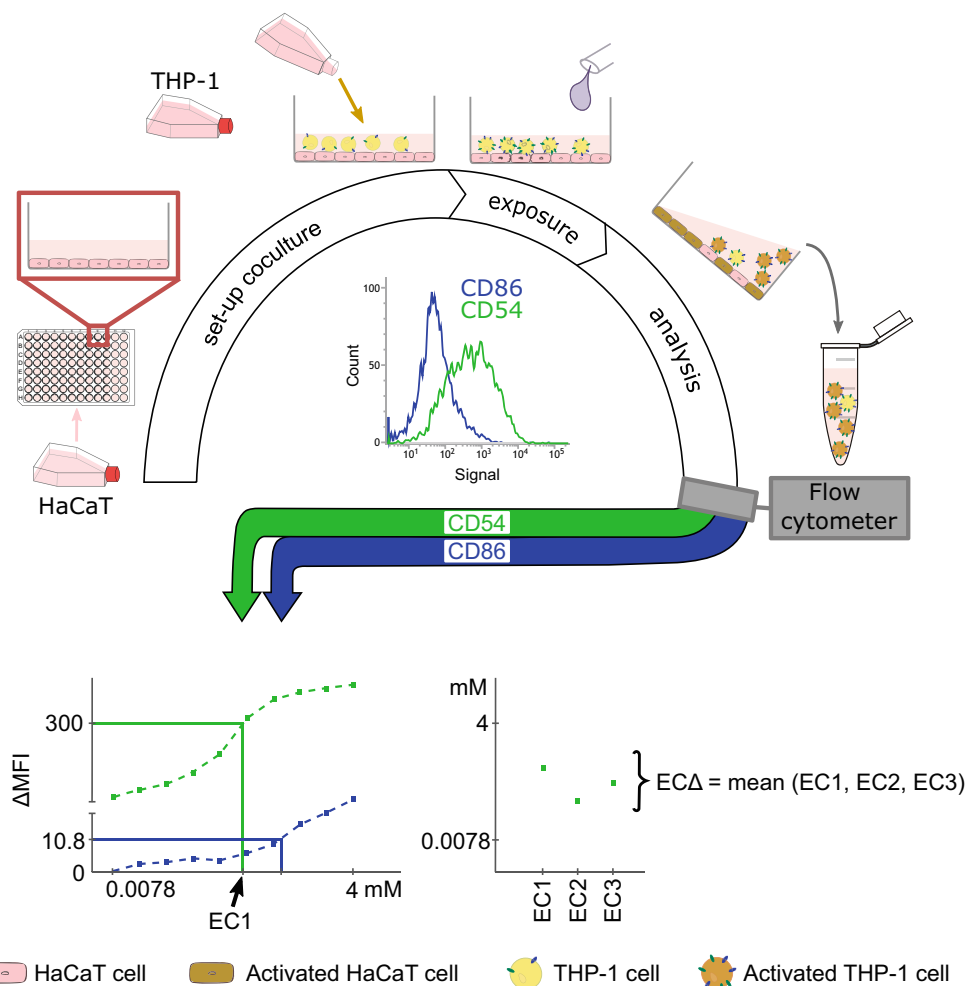
3 | RESULTS

3.1 | Evaluation of the method

The current study focused on the DC activation in the presence of keratinocytes testing a set of fragrance chemicals and related

chemicals. Representative flow cytometry histogram plots of solvent, geraniol and geranial are shown in Figure 2. Representative concentration–effect curves of the experimental runs with geraniol and geranial are depicted in Figure 3.

A summary of all substances considered is reported in Table 1. The 27 test chemicals covered typical classes of fragrance materials and comprised most of the fragrance chemicals requiring labelling in the European Union. With the exception of the synthesized substances, chemicals were of the quality provided by commercial sources, thus ensuring that data generated using them were relevant to the real-life situation. Eleven chemicals are assigned to the group of pre/prohaptens, that are suspected to be more difficult to be identified in vitro, but also using other NAMs.^{17,25} Table 1 reports also on the maximal concentrations resulting in more than 50% THP-1 viability. Although these concentrations varied between chemicals (0.125–4 mM), we still could test between 5 and 10 concentrations of each chemical. In principle, a chemical can upregulate either no marker, one or both markers (CD86, CD54) on the cell surface of cocultured THP-1 cells. For each substance and experimental runs, we listed the lowest concentration reaching marker positivity. In detail, CD86 was responsible for positivity in 59 of 86 runs and CD54 in 37 of 86 performed experimental runs, respectively. Therefore, CD86 was considered the more sensitive marker for this set of chemicals.



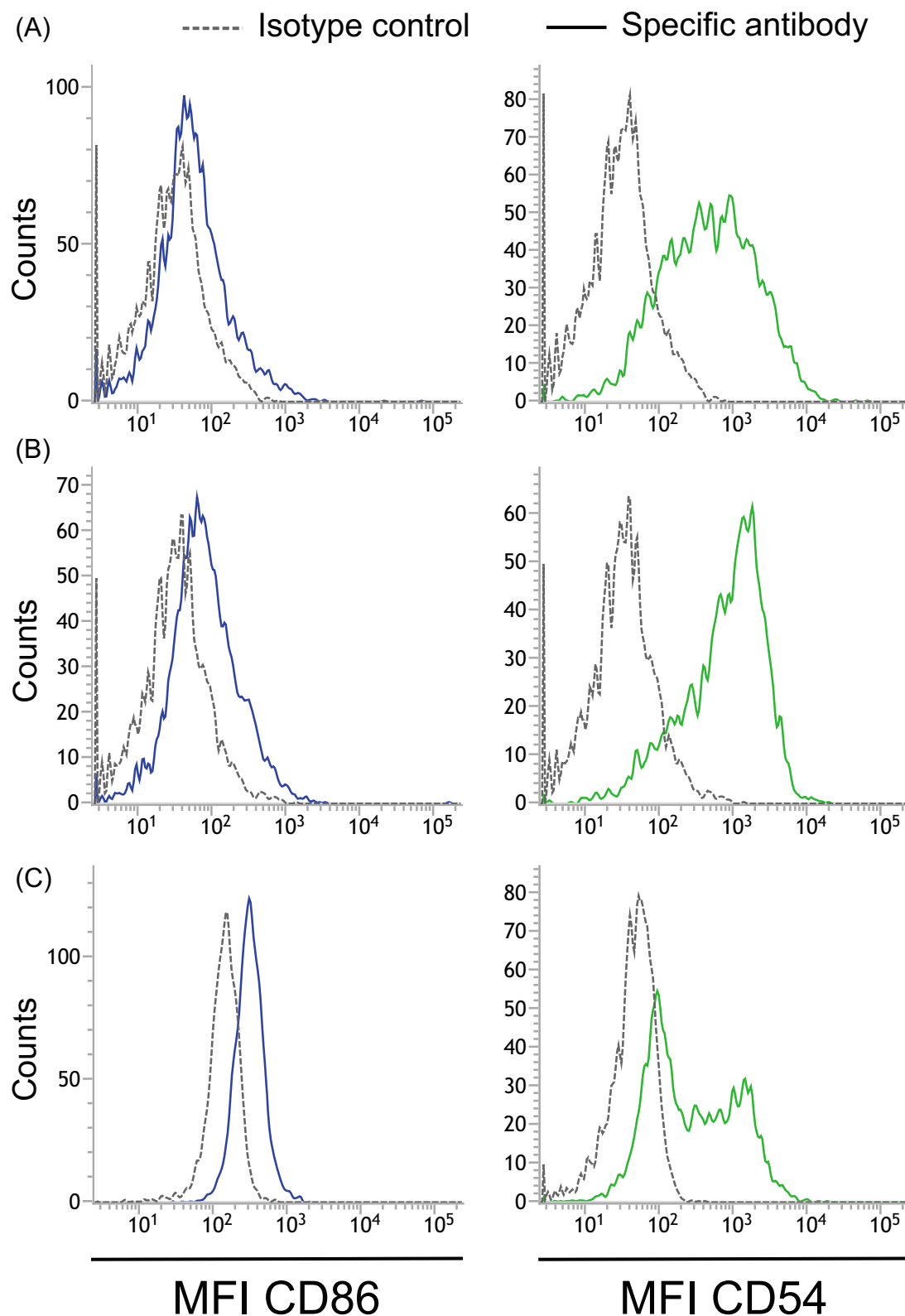


FIGURE 2 Representative flow cytometry histograms for the geometric mean fluorescence intensity (MFI) of CD86 and CD54. (A–C) Expression of CD86 and CD54 on cocultured THP-1 cells after treatment with solvent 0.2% dimethylsulfoxide (A), 2 mM geraniol (B) or 0.125 mM geraniol (C). Flow cytometry histograms show the number of events (cell counts) versus fluorescein isothiocyanate (FITC) (CD86) and allophycocyanin (APC) (CD54) MFI. Solid lines represent histograms for the indicated antibodies (FITC-labelled anti-CD86 or APC-labelled anti-CD54), and dotted lines represent histograms of the corresponding isotype controls (FITC-labelled or APC-labelled IgG1), respectively.

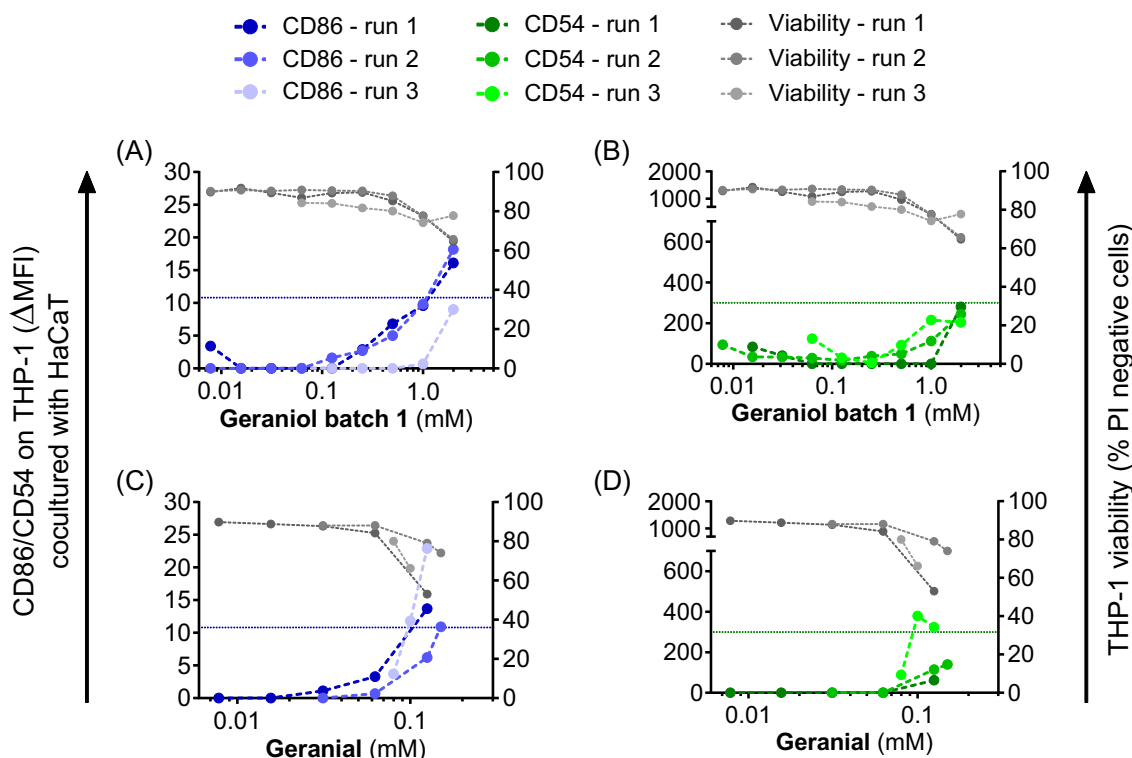


FIGURE 3 Concentration-dependent upregulation of CD86 and CD54 on cocultured THP-1 cells in the COCAT method. THP-1 cells cocultured with HaCaT keratinocytes were exposed to geraniol (batch 1; A, B) and geraniol (C, D) for 24 h. The expression of CD86 and CD54 was analysed on harvested viable THP-1 cells (propidium iodide negative) with flow cytometry, and given as chemical-induced differences (Δ MFI) in cell surface levels of CD86 or CD54 of the individual runs. The blue and green dotted horizontal lines represent the threshold for positivity of CD86 and CD54 with a Δ MFI of 10.8 and 300, respectively.

When a test chemical upregulated no marker despite testing all 10 concentrations, results were designated negative at 4 mM. Overall, the results of individual runs indicated good repeatability. For two substances (geraniol, citral) we repeated the entire testing procedure on two batches to confirm the good performance of COCAT. No major differences were noted. Exceptions were noted for (R)-(-)-carvone, coumarin and limonene. For example, a significant variation between runs was observed for limonene ($EC_1 = 1.541$ mM, $EC_2 = 1.710$ mM, $EC_3 = 0.157$ mM). Interestingly, positivity of geraniol was based on CD86, while its oxidation products geranial, neral and the mixture thereof (citral, 60:40) upregulated both markers.

Looking at individual chemicals, we found that the positive runs in case of anisyl alcohol, benzyl alcohol, coumarin, geraniol (batch 1 and 2), geraniol-6/7-hydroperoxide, α -isomethylone, and 2-methoxy-4-methylphenol solely relied on CD86 ($n = 7$), while only CD54 positive runs were found for benzaldehyde, benzyl cinnamate, hydroxycitronellol, limonene and linalool ($n = 5$). For chemicals that upregulated both markers ($n = 14$), we observed for nine chemicals that CD86 reached the positivity threshold at lower concentrations than CD54 ($n \geq 2$ runs), and CD86 was therefore considered the more sensitive marker for those chemicals. Considering defined criteria of positivity, 24 of 27 tested substances were tested positive (≥ 2 out of 3 runs). 1-Octen-3-yl-acetate and aryl aldehydes benzaldehyde and vanillin, that rarely cause allergic reactions in patients, were tested

negative. In the case of benzaldehyde, it should be noted, that the chemical upregulated CD86 but reached positivity only in one run, while 1-octen-3-yl-acetate and vanillin did not show any upregulation of CD86 or CD54. In addition, all pre- and/or prohaptens were identified correctly.

A graphical overview of the chemicals' effective concentration (EC_{Δ} , see also Material and method section and Figure 1) is shown in Figure 4. The EC_{Δ} value of a chemical is taken as an indicator of its potency. Overall, EC_{Δ} values varied between 3.9 and 0.091 mM, demonstrating a good dynamic range of the method considering that fragrance allergens are known to express varying degrees of sensitization potency.^{26–28} Further, EC_{Δ} values indicated distinct differences. The highest EC_{Δ} values, indicative of lower potency, were found for the two tested benzyl alcohols anisyl alcohol and benzyl alcohol. In contrast, the α,β -unsaturated aldehydes^{29,30} *trans*-2-hexenal, cinnamaldehyde, geranial and neral gave the lowest EC_{Δ} values in our set, ranging between 0.091 and 0.12 mM. Among the alcohols, anisyl alcohol was the weakest alcohol (EC_{Δ} : 3.92 mM), while EC_{Δ} values of hydroxycitronellol and geraniol were 1.6 mM and 1.5 mM (mean of the two tested batches), respectively. COCAT EC_{Δ} values of fragrance chemicals with ester groups, namely coumarin and benzyl cinnamate ranged between 1 and 1.9 mM. COCAT EC_{Δ} values of citronellol, linalool, limonene and geraniol varied between 0.53 and 1.1 mM (linalool $\approx$ citronellol < limonene $\approx$ geraniol). Furthermore,

TABLE 1 Reproducibility of upregulation of CD86 and CD54 in COCAT.

Fragrance chemicals and related chemicals	Pre-/Pro-hapten ^{17,25}	Solvent	Maximum concentration (mM) inducing a >50% THP-1 cell viability	No. of tested concentrations	Lowest concentration (mM) inducing CD86 positivity with Δ MFI ≥ 10.8			Lowest concentration (mM) inducing CD54 positivity with Δ MFI ≥ 300		
					1st run	2nd run	3rd run	1st run	2nd run	3rd run
Anisyl alcohol		DMSO	4	10	-	3.98	3.87	-	-	-
Benzaldehyde		DMSO	4	10	-	-	-	-	-	0.56
Benzyl alcohol	Pro	DMSO	4	10	3.84	3.77	-	-	-	-
Benzyl cinnamate		DMSO	2	9	-	-	-	2.61	-	1.15
(R)-(-)-Carvone		DMSO	2	9	0.692	2.0	-	0.695	0.799	0.312
Cinnamaldehyde		DMSO	0.25	6	0.157	0.167	0.134	0.130	0.164	0.127
Cinnamyl alcohol	Pre/Pro	DMSO	1	8	0.797	1.12	0.899	0.872	0.971	0.809
Citral (Geraniol/Neral), batch 1		DMSO	0.25	6	0.112	0.110	-	-	-	0.118
Citral, batch 2		DMSO	0.125	5	0.107	0.104	^a	0.110	0.117	^a
Citronellol	Pre	DMSO	1	8	-	0.998	0.424	0.530	-	-
Coumarin ^b	Pre/Pro	DMSO	4	10	0.218	1.210	1.462	-	-	-
Eugenol ^b	Pre/Pro	DMSO	1	8	0.433	0.443	0.438	0.386	0.440	0.620
Geraniol ([2E]-3,7-Dimethyl-2,6-octadienal)		DMSO	0.25	6	0.108	0.15	0.98	-	-	0.74
Geraniol, batch 1	Pre/Pro	DMSO	2	9	1.184	1.301	1.121	-	-	-
Geraniol, batch 2	Pre/Pro	DMSO	2	9	1.90	-	1.726	-	-	-
Geraniol-6/7-hydroperoxide		DMSO	0.25	6	0.148	0.204	0.174	-	-	-
trans-2-Hexenal (E-hex-2-enal)		DMSO	0.5	7	0.93	0.109	0.94	0.69	-	0.111
Hydroxycitronellal		DMSO	4	10	0.158	0.183	0.185	0.131	0.164	0.152
Hydroxycitronellol		DMSO	4	10	-	-	-	2.027	1.099	-
4-(4-Hydroxy-4-methylpentyl) cyclohex-3-ene-1-carbaldehyde (HICC)		DMSO	0.5	7	0.151	0.243	0.133	0.191	-	0.212
Isoeugenol ^b	Pre	DMSO	0.5	7	0.364	0.544	0.495	0.282	0.396	0.410
Isosomethylionone		DMSO	0.25	6	-	0.174	0.154	-	-	-
Limonene	Pre	DMSO	4	10	-	-	-	1.541	1.710	0.157
Linalool ^b	Pre	DMSO	4	10	-	-	-	0.279	-	0.781
2-Methoxy-4-methylphenol	Pre/Pro	DMSO	0.5	7	0.507	0.480	0.721	-	-	-
Methyl 2-octynoate (Methyl heptene carbonate)		DMSO	4	10	1.167	1.961	0.675	-	-	1.995
Neral ([2Z]-3,7-dimethyl-2,6-octadienal)		DMSO	0.25	6	0.133	0.131	0.102	-	-	0.095

TABLE 1 (Continued)

Fragrance chemicals and related chemicals	Pre-/Pro-hapten ^{17,25}	Solvent	Maximum concentration (mM) inducing a >50% THP-1 cell viability	No. of tested concentrations	Lowest concentration (mM) inducing CD86 positivity with Δ MFI ≥ 10.8			Lowest concentration (mM) inducing CD54 positivity with Δ MFI ≥ 300		
					1st run	2nd run	3rd run	1st run	2nd run	3rd run
1-Octen-3-yl acetate		DMSO	4	10	-	-	-	-	-	-
Vanillin		DMSO	4	10	-	-	-	-	-	-

Note: The table provides the assignment of the substance (alphabetical order) to the group of pre-/prohaptens, the maximum concentration inducing more than 50% viability of THP-1 cells after 24 h (acceptance criteria in COCAT), resulting total number of tested concentrations, and the lowest concentration of a chemicals inducing CD86 or CD54 above the prediction model thresholds, Δ MFI ≥ 10.8 for CD86 and Δ MFI ≥ 300 for CD54. Concentrations between 4 and 0.0078 mM (4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156 and 0.0078) in the solvent 0.2% dimethylsulfoxide were tested. The lowest concentration reaching positivity for CD86 or CD54 was calculated by linear interpolation for each run. Bold values indicate the lowest effective concentration of a run. '-' indicates that none of the tested concentrations exceeded the respective threshold.

Abbreviations: DMSO, dimethylsulfoxide; Pre, prehapten; Pro, prohapten.

^aNot available.

^bAlso tested during protocol optimization.¹⁶

comparison of the results of limonene with (R)-(-)-carvone, one of its oxidation products, found a clear difference. Limonene had a twofold higher EC Δ (1.1 mM) than (R)-(-)-carvone (0.6 mM). Similarly, geraniol showed higher EC Δ values (batch 1 and 2) compared to its main oxidations products as discussed in Section 3.2.

COCAT EC Δ values ($\pm$ mean) of the tested substances and available in vivo data are reported in Table 2, along with their structure and CAS number to ensure proper definition of the chemical studied. Data on in vivo skin sensitization potencies of chemicals are given for comparison. These data were generated by experts in the field to assist those working to produce new NAMs, capable of predicting the relative human potency of skin sensitizing substances. Basketter and Api^{26,31} approached the subcategorization of sensitizing substances solely on the basis of human data. They used primarily information based on the Human Repeat Insult Patch Test (HRIPT) information (No Observed Effect Levels, NOEL HRIPT), backed up by Human Maximization Test (HMT)³² data, if available. Further refinement of this information is based on diagnostic patch test results, namely frequency of positive patch tests from multiclinic-collated data of consecutive patients with eczema, and also consideration of the production volume of a chemical. For completeness, although not relevant for our substances, in a few instances (e.g., hexyl tiglate, CAS no. 16930-96-4) HRIPT information was not given the priority as HMT information suggested that the HRIPT NOEL is too low. Totally 6 classes are nominated: category 1 (extreme; induction level <25 μ g/cm²; patch test frequency >3%), 2 (strong; induction level 25–500 μ g/cm²; patch test frequency >1%), 3 (moderate; 500–2500 μ g/cm²; patch test frequency up to 1%), 4 (weak; >2500 μ g/cm²; patch test frequency less than category 3), 5 (very weak; >10 000 μ g/cm²; rarely reported except selected groups) and 6 (non-sensitizer; meaning that a high test concentration yielded no evidence of the induction of skin sensitization, absence of positives despite testing).

For 16 fragrance chemicals, we listed potency values (PV; given in μ g/cm²).³³ PV are also based on expert judgement upon consideration of local lymph node assay (LLNA) data and relevant human data, thereby taken into account reported differences between LLNA and humans.²⁶ PV provide continuous values of skin sensitizing potency against which the accuracy of predictions can be judged. Comparison of COCAT EC Δ with in vivo data found similarities and few differences (Table 2). For instance, COCAT EC Δ of methyl-2-octynoate is 1.3 mM. Knowing its in vivo potency (category 2, PV value of 125 μ g/cm²) we aimed to ensure the reliability of our results by independent repetition of the experimental runs, again confirming the initial EC Δ value. Similar to in vivo, isoeugenol (0.36 mM) and structurally related fragrance chemicals 2-methoxy-4-methylphenol (0.57 mM) and eugenol (0.42 mM), were correctly ranked (isoeugenol >2-methoxy-4-methylphenol/eugenol).

3.2 | Investigation of fragrance chemicals susceptible to oxidative activation in the COCAT

We also tested fragrance chemicals that are known for their oxidation potentials. The resulting oxidations products are typically

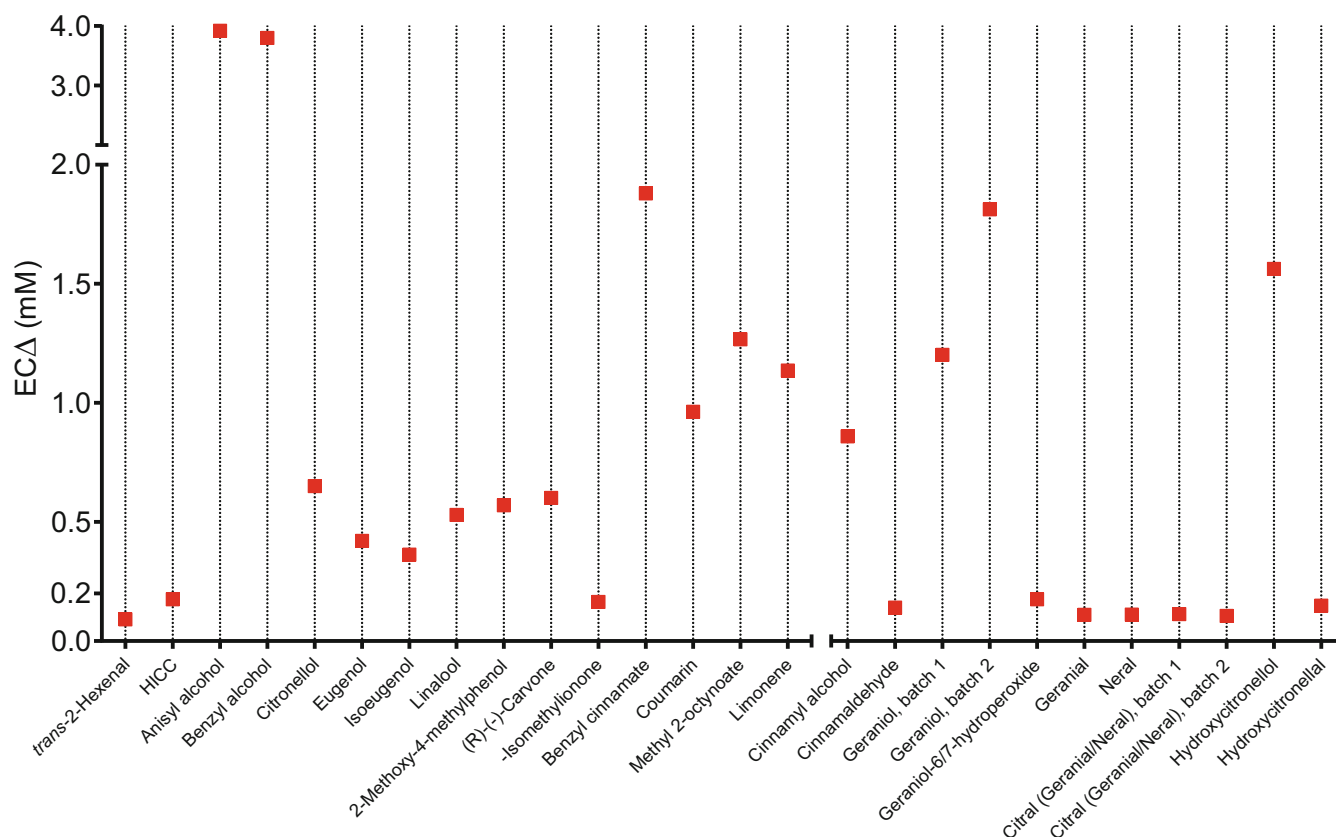


FIGURE 4 Compilation of positive tested fragrance chemicals and related chemicals with the effective concentration in COCAT (ECΔ in mM). Tested chemicals are arranged according to their chemical structure and within this group according to the alphabet: aldehydes, alcohols, ketones, esters and terpenes. Pairwise comparison of alcohols with corresponding aldehydes is shown after a break of the x-axis. Geraniol/geranial pair analysis is complemented with different batches, geraniol-6/7-hydroperoxide, Z-isomer neral and E-Z-mixture in citral. HICC, 4-(4-hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde.

much stronger sensitizers *in vivo* than the parent substances.^{34,35} As example, in the oxidation mixture of limonene were important contact allergens identified, namely its oxide, (R)-(-)-carvone, and limonene-1- and -2-hydroperoxide.^{34,35} As expected from *in vivo* data, limonene (category 5), showed the highest ECΔ (1.14 mM) followed by (R)-(-)-carvone with a ECΔ = 0.6 mM (Table 2). Further, geraniol (1.5 mM, mean of batch 1 and 2) showed a 10-fold difference in ECΔ values compared to its oxidation products geraniol-6/7-hydroperoxides (0.175 mM), geranial (0.11 mM), and neral (0.11 mM). Looking at alcohols and corresponding aldehyde pairs, higher ECΔ values were observed for the alcohols compared to the aldehydes, with ECΔ values differing 6- to 13-fold. Specifically, cinnamaldehyde (0.14 mM) and cinnamyl alcohol (0.86 mM) differed by sixfold, the difference between citral (0.110 mM, mean of batch 1 and 2) and geraniol (1.5 mM) was 13-fold, and 11-fold between hydroxycitronellal (0.15 mM) and hydroxycitronellol (1.6 mM). Summing up, apart from the well-known exception benzyl alcohol/benzaldehyde, pair comparison of not easily accessible chemicals has enabled a first prediction of missing chemical functionality in this assay.

4 | DISCUSSION

4.1 | Evaluation of the COCAT method and purity of chemicals

In order to increase our knowledge about fragrance chemicals' potential to activate and mature DC in their environment we evaluated a set of common fragrances and related chemicals in a 2D coculture model. Closer to the *in vivo* situation, our 2D coculture method (COCAT) contains keratinocytes next to DC (THP-1) allowing for direct cell-cell communication, metabolism and exchange of biological materials (proteins, enzymes, lipids, mRNA, vesicles). Previously, we demonstrated keratinocytes' potentials for supplementation of targets for DC activation, by comparing results of chemicals in the absence and presence of HaCaT keratinocytes using THP-1 cells or monocyte-derived DC. In these studies, we found that keratinocytes can modulate the strength of chemical-induced DC activation by providing both metabolic activation but also conjugation capacities for chemicals.¹²⁻¹⁴ For example, enzymes known to transform fragrance chemicals like esters, aldehydes and alcohols are present in COCAT

TABLE 2 Compilation of COCAT ECΔ (mM), mean ± SEM of fragrance chemicals and related chemicals (solvent 0.2% dimethylsulfoxide) and chemicals tested negative at 4 mM.

Fragrance chemicals and related chemicals	CAS no.	Molecular structure	Chemical structure class(es)	Relative human skin sensitization potency class (1–6) ^{8,26,31}	PV (μg/cm ²) ³³	COCAT ECΔ (mM), mean ± SEM; negative at 4 mM ¹⁶
<i>trans</i> -2-Hexenal (E-hex-2-enal)	6728-26-3		α,β-Unsaturated aldehyde	2	39.9	0.091 ± 0.012
Citral (Geranial/ Neral, 60:40), batch 2 (n = 2 runs)	5392-40-5		α,β-Unsaturated aldehyde	3	1450	0.106
Geranial ([2E]-3,7-Dimethyl-2,6-octadienal)	141-27-5		α,β-Unsaturated aldehyde	^a	^a	0.11 ± 0.02
Neral ([2Z]-3,7-dimethyl-2,6-octadienal)	106-26-3		α,β-Unsaturated aldehyde	^a	^a	0.11 ± 0.01
Citral (Geranial/ Neral, 60:40), batch 1	5392-40-5		α,β-Unsaturated aldehyde	3	1450	0.113 ± 0.002
Cinnamaldehyde	104-55-2		α,β-Unsaturated aldehyde	2	885	0.14 ± 0.01
Hydroxycitronellal	107-75-5		Aldehyde; tertiary alcohol	4	5275	0.15 ± 0.02
α-Isomethylionone	127-51-5		α,β-Unsaturated ketone	5	>25 000	0.16 ± 0.01
Geraniol-6/7-hydroperoxide	85867-61-4/ 85867-60-3		α,β-Unsaturated alcohol; α,β-unsaturated hydroperoxide	^a	^a	0.175 ± 0.016
4-(4-Hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde (HICC)	31906-04-4		Aldehyde; tertiary alcohol	2	4275	0.18 ± 0.04
Isoeugenol	97-54-1		Aryl alcohol; alkyl aryl ether	2	325	0.36 ± 0.04
Eugenol	97-53-0		Aryl alcohol; alkyl aryl ether	3	7357	0.42 ± 0.02

(Continues)

TABLE 2 (Continued)

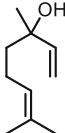
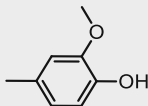
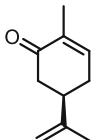
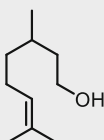
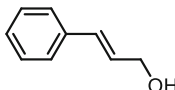
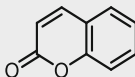
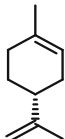
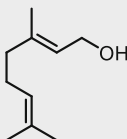
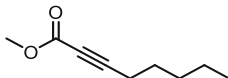
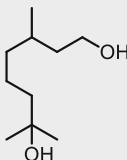
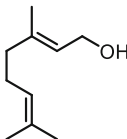
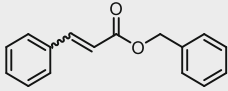
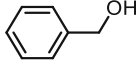
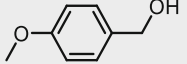
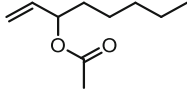
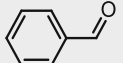
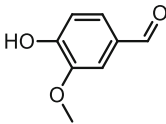
Fragrance chemicals and related chemicals	CAS no.	Molecular structure	Chemical structure class(es)	Relative human skin sensitization potency class (1–6) ^{8,26,31}	PV ($\mu\text{g}/\text{cm}^2$) ³³	COCAT EC Δ (mM), mean $\pm$ SEM; negative at 4 mM ¹⁶
Linalool	78-70-6		α,β -Unsaturated tertiary alcohol	4	^a	0.53 $\pm$ 0.25
2-Methoxy-4-methylphenol	93-51-6		Aryl alcohol; alkyl aryl ether	2	^a	0.57 $\pm$ 0.08
(R)-(-)-Carvone	6485-40-1		α,β -Unsaturated ketone	4	17 573	0.60 $\pm$ 0.15
Citronellol	106-22-9		Primary alcohol	5	^a	0.65 $\pm$ 0.18
Cinnamyl alcohol	104-54-1		α,β -Unsaturated alcohol	3	5775	0.86 $\pm$ 0.05
Coumarin	91-64-5		α,β -Unsaturated ester (lactone)	3	11 792	0.96 $\pm$ 0.38
Limonene	5989-27-5		Monocyclic monoterpene	5	^a	1.1 $\pm$ 0.5
Geraniol (batch 1)	106-24-1		α,β -Unsaturated alcohol	4	9197	1.20 $\pm$ 0.04
Methyl 2-octynoate (Methyl heptene carbonate)	111-12-6		α,β -Unsaturated ester	2	125	1.3 $\pm$ 0.4
Hydroxycitronellol	107-74-4		Primary and tertiary alcohol	^a	^a	1.6 $\pm$ 0.7
Geraniol (batch 2)	106-24-1		α,β -Unsaturated alcohol	4	9197	1.8 $\pm$ 0.1

TABLE 2 (Continued)

Fragrance chemicals and related chemicals	CAS no.	Molecular structure	Chemical structure class(es)	Relative human skin sensitization potency class (1–6) ^{8,26,31}	PV (µg/cm ²) ³³	COCAT ECA (mM), mean ± SEM; negative at 4 mM ¹⁶
Benzyl cinnamate	103-41-3		α,β-Unsaturated ester	4	^a	1.9 ± 0.7
Benzyl alcohol	100-51-6		Benzyl alcohol	4	>25 000	3.80 ± 0.05
Anisyl alcohol	105-13-5		Benzyl alcohol; alkyl aryl ether	4	^a	3.92 ± 0.05
1-Octen-3-yl acetate	2442-10-6		Ester	4	^a	Negative
Benzaldehyde	100-52-7		Aryl aldehyde	5	4094	Negative
Vanillin	121-33-5		Aryl aldehyde; alkyl aryl ether; aryl alcohol	5	Very weak, non-sensitizer	Negative

Note: Tested chemicals are listed according to ascending ECA values. Chemicals are further characterized by corresponding CAS no., molecular structure, chemical structure class, relative human skin sensitization potency class (categories 1–6),^{8,26,31} and potency value (µg/cm²),³³ if available. Relative human skin sensitization potency classes are built on the Human Repeat Insult Patch Test information and further refinement based on diagnostic patch test results in combination with production volume. Classes nominated: category 1 (extreme; induction level <25 µg/cm², patch test frequency >3%), 2 (strong; induction level 25–500 µg/cm², patch test frequency >1%), 3 (moderate; 500–2500 µg/cm², patch test frequency up to 1%), 4 (weak; >2500 µg/cm², patch test frequency less than cat. 3), 5 (very weak; >10 000 µg/cm², rarely reported except selected groups) and 6 (non-sensitizer; meaning that a high-test concentration yielded no evidence of the induction of skin sensitization, absence of positive patch test results despite testing). Potency values give continuous values as measure of skin sensitizing potency based on expert judgement upon consideration of relevant human data and local lymph node assay data.

Abbreviation: PV, potency value.

^aNot available.

and may influence ECA values. This assumption is based on data demonstrating that monocultured HaCaT cells possess aldehyde dehydrogenase activity, which is not the case for THP-1 cells. On the other hand, alcohol dehydrogenase activity was not detected in HaCaT and primary keratinocytes using ethanol as substrate, and only occasionally detected in THP-1 cells.³⁶ Studying the impact of coculture on the N-acetyltransferase 1 activities for acetylation of para-aminobenzoic acid we found that activities behaved additively (unpublished data). In contrast, for 7-ethylresorufin-O-deethylase (EROD) activity in the presence of eugenol we demonstrated that eugenol significantly induces EROD activity in HaCaT cells, and the response was even enhanced after coculture with THP-1 cells.¹⁴ After optimization and training of COCAT with different chemicals, we demonstrated COCAT's predictive capacity to both skin sensitization hazard and potency sub-categorization according to the UN GHS classification based on the blind testing of 15 coded chemicals. Results showed good reproducibility between runs, high sensitivity and accuracy.¹⁶

In the present study, testing of a set of 27 fragrance chemicals or related substances covering different classes (alcohols, aldehydes, ketones, esters), mode of action, and a wide range of sensitizing potency we showed that all three very weak contact allergens were correctly identified. Although volatility and hydrophobicity of some of the fragrance chemicals could have influenced in vitro testing of chemicals, reasonable potencies (ECA values) were achieved. For coumarin and limonene we noticed a lower reproducibility of the EC values in one of three runs (see Table 1). Noteworthy, both belong to the group of sensitizers that have been assigned as pre- and/or prohap- tens and reported to give somewhat more variable results as the rate of oxidation may vary between runs.^{25,37} Overall, the COCAT method has been proven to be a sensitive method able to detect weak sensitizers, also among chemicals^{23,38} which can be difficult to assess in in vitro assays. However, the results are dependent upon careful monitoring of the purity of the test chemicals used. It has previously been shown that oxidation products can be present at delivery of chemicals prone to autoxidation.^{39,40} Thus, careful monitoring of the purity of

test materials used in NAMs is of great importance in order to achieve reliable results.

4.2 | COCAT results compared to in vivo data

The determination of the sensitizers' potency quantitatively is critical for risk assessment, especially for consumer and occupational exposures. However, the assessment of skin sensitizing potency is very challenging.^{41,42} Although direct comparison of COCAT ECΔ values (mM) with in vivo data such as the Relative Human Skin Sensitization Potency Classes^{26,31} or Potency Values (PV in $\mu\text{g}/\text{cm}^2$) as measure of skin sensitizing potency³³ is not possible, the data can be used for ranking of chemicals.

Three test substances were tested negative. Assignment of 1-octen-3-yl-acetate to category 4 (Relative Human Skin Sensitization Potency Class) indicates that 1-octen-3-yl-acetate is false negative in COCAT. The other two negative tested molecules, the aryl aldehydes benzaldehyde and vanillin belong to the group of very weak sensitizers that rarely causing positive diagnostic patch test results in patients (category 5).

Looking at the chemicals capacity to induce positivity (see also Table 2), we noted that the majority of the fragrance chemicals that induced upregulation of only one marker, either CD86 or CD54 ($n = 13$), are weak or very weak human sensitizers. Looking at the Relative Human Skin Sensitization Potency Class, nine of the 11 chemicals inducing only positivity of one marker are belonging to either category 4 ($n = 6$) or 5 ($n = 3$). Exceptions were found among those chemicals, that solely upregulated CD86, namely coumarin (category 3) and 2-methoxy-4-methylphenol (category 2). This data suggest that chemicals that upregulate only one marker and especially CD54 are likely weaker sensitizers.

Surprisingly, limonene (ECΔ = 1.16 mM), citronellol (ECΔ = 0.65 mM) and linalool (ECΔ = 0.53 mM) that are also assigned to class 5 and class 4 were tested positive with distinct ECΔ values. In animal studies, their oxidation products are potent sensitizers, specifically hydroperoxides were identified as key drivers of skin allergy. In addition, analytical measurement of the sample quality of these substances used for experiments and in vivo testing under low level of oxidation demonstrated, that only purified substances are non-sensitizers.^{34,43,44} As limonene, citronellol and linalool were not purified before testing, we cannot exclude that they contained oxidation products like hydroperoxides. In addition, our previous evaluation of the pure hydroperoxides of limonene, citronellol and linalool in COCAT gave results that are lower than for the parent chemicals comparable with their sensitization potency ranking in the LLNA, used for comparison due to absence of predictive human data.¹⁵ Comparison of results of (R)-(–)-carvone (ECΔ = 0.60 mM) with limonene-2-hydroperoxide (ECΔ = 0.35 mM)¹⁵ and results of limonene (ECΔ = 1.5 mM) shows clear differences. The somewhat smaller ECΔ difference between limonene-2-hydroperoxide (0.35 mM) and (R)-(–)-carvone (0.60 mM) may be explained by mechanistic studies showing that limonene-2-hydroperoxide can react further by elimination of water to give carvone.⁴⁵ In line, (R)-(–)-carvone and

similar α,β -unsaturated ketones, that gain protein reactivity as Michael acceptors, are typically less potent in vivo than the hydroperoxides.⁴⁶ Comparison of ECΔ values of (R)-(–)-carvone or the α,β -unsaturated ketone α -isomethylionone (ECΔ = 0.16 mM) to the in vivo data (see Table 2), suggest that COCAT may be very sensitive to these chemical structure classes.

Our test set contained 16 fragrance chemicals of the 33 Reference Chemical Potency List curated to rank chemicals according to their potency.³³ These values are based on expert judgement upon consideration of relevant human data and LLNA data, thereby taking into account reported differences between information of the LLNA and humans.²⁶ Global comparison with the ECΔ values of this study found reasonable trends. In principle, COCAT method could distinguish strong and moderate sensitizer from weak and very weak fragrance chemicals. Clearly, α,β -unsaturated ketones with integration into a six-ring (carvone) or the complex, sterically demanding side groups (α -isomethylionone) seemed overpredicted. Ester functionality, as the structural element in methyl 2-octynoate and coumarin (lactone, cyclic ester) seemed underestimated, while esters like benzyl cinnamate or 1-octen-3-yl acetate compare better.

4.3 | COCAT results and chemical families

Comparing alcohols and their corresponding aldehydes, the aldehyde is many times the stronger sensitizer, reflected by the provided PV values and categories. Allylic and conjugated alcohols are further known to be easily oxidized to aldehydes as well as hydroperoxides. Among the identified oxidation products, hydroperoxides represent the strongest sensitizer based on LLNA data.^{23,35,40,44,47} Focusing on the COCAT ECΔ value as an indicator for potency, we found clear differences between alcohols and corresponding aldehyde pairs (6- to 13-fold, see Table 2). Specifically, a sixfold difference was observed for cinnamaldehyde and cinnamyl alcohol. There are several reasons for the smaller difference compared to other pairs. First, cinnamyl alcohol is known to oxidize rapidly upon air exposure, and commercially available cinnamyl alcohol may be contaminated with cinnamaldehyde and sensitizing epoxides.^{40,47} This could explain the smaller difference between cinnamyl alcohol and aldehyde compared to the other unsaturated alcohols. Furthermore, patch test studies have found individuals, sensitized to cinnamyl alcohol who do not react to the aldehyde. This data and other publications demonstrate that alcohols and corresponding aldehydes can act as separate haptens and that metabolic activation of these alcohols shows donor-dependency.^{22,48}

In contrast to geraniol, geranial and neral were purified using distillation before use in order to prevent contamination from oxidation products. Testing in COCAT resulted in a 13-fold difference (see Table 3). Data ranking was found to be in agreement with data from animal studies (LLNA). Human Potency classes and PV values are not available for this set of chemicals. For the two isomers geranial and neral and the binary mixture (60:40) citral, we found no differences. This result is a bit different from LLNA and human patch testing

TABLE 3 Comparison of in vitro COCAT (mM) results of geraniol and oxidation products and in vivo (local lymph node assay) results.

Fragrance chemicals and related chemicals	LLNA EC3 (M; % wt/vol)	COCAT ECΔ (mM)
Geraniol	1.45; 22.4	1.5 ^a
Geranial	0.45; 6.8	0.11
Neral	0.64; 9.7	0.11
Citral (Geranial/Neral, 60:40)	0.40; 6.1	0.110 ^a
Geraniol-6/7-hydroperoxide	0.07; 1.4	0.175

Abbreviation: LLNA, local lymph node assay.

^aMean of batch 1 and 2.

results, where neral shows slightly lower frequencies of contact allergy in dermatitis patients; however, this difference was not statistically significant.^{49,50} The latter again clearly indicate that geranial and neral, although closely chemically related, can act as separate haptens.³⁸ In addition, our results argue against one hypothesis that geraniol is efficiently oxidized to geranial in humans and postulated cause of observed contact allergy to geraniol. The difference is also in line with clinical results, where concomitant patch testing with citral and geraniol showed higher frequencies of contact allergy to citral and low concomitant reactivity to geraniol.^{38,49} Interestingly, the ECΔ value of geraniol-6/7-hydroperoxides is comparable to the two aldehydes of geraniol. This differs from LLNA and patch test studies, where a difference between geranial/neral and the geraniol-6/7-hydroperoxides was seen.^{23,38} However, the ECΔ value of geraniol-6/7-hydroperoxides is comparable to previously reported COCAT ECΔ values of hydroperoxides of limonene, linalool and citronellol.¹⁵

Hydroxycitronellal and hydroxycitronellol differed by 11-fold. This finding is also in line with patch test studies demonstrating that the two molecules can act as separate allergens.⁵¹ Summarizing our data for the tested aldehydes we received ECΔ values as low as 0.091 mM (*trans*-2-hexenal). On the other end ranked two aryl aldehydes, namely the extremely weak allergens benzaldehyde and vanillin, which appeared negative in COCAT at maximal tested concentration (4 mM). These results are comparable to in vivo assays,²⁹ finding for aldehydes a wide range of EC3 values (<1 up to 50%), covering potent allergens and non-sensitizing molecules.

In conclusion, the results provide DC activation by fragrance chemicals and related oxidation products exposed in their natural environment of human keratinocytes. This 2D approach is closer to the in vivo exposure scenario than using DC alone. A wide selection of fragrance chemicals and related chemicals could be characterized in 2D coculture and correctly identified. Furthermore, binary mixtures of isomers and chemical functionality may be analysed in this model. In sum, testing fragrance chemicals in this in vitro method based on human cells may provide better understanding of the frequently occurring skin reactions to fragrance chemicals, and COCAT can help in the assessment of potential and potency of sensitizing chemicals in humans.

AUTHOR CONTRIBUTIONS

Niklas Peter: Data curation; formal analysis; methodology; writing – original draft; writing – review and editing. **Jutta Lichter:** Formal analysis; methodology; visualization; writing – review and editing. **Lina Hagvall:** Formal analysis; resources; writing – review and editing. **Udo Bock:** Conceptualization; funding acquisition; resources; formal analysis; writing – review and editing. **Brunhilde Blömeke:** Writing – original draft; writing – review and editing; project administration; funding acquisition; supervision; formal analysis; conceptualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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