



Chemical stability and *in chemico* reactivity of 24 fragrance ingredients of concern for skin sensitization risk assessment

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ABSTRACT

Twenty-four pure fragrance ingredients have been identified as potential concern for skin sensitization. Several of these compounds are chemically unstable and convert into reactive species upon exposure to air or light. In the present work, a systematic investigation of the correlation between chemical stability and reactivity has been undertaken. The compounds were subjected to forced photodegradation for three months and the chemical changes were studied with GC–MS. At the end of the stability study, two-thirds of the samples were found to be unstable. The generation of chemically reactive species was investigated using the *in chemico* HTS-DCYA assay. Eleven and fourteen compounds were chemically reactive before and after three months, respectively. A significant increase in reactivity upon degradation was found for isoeugenol, linalool, limonene, lylal, citronellol and geraniol; in the same conditions, the reactivity of hydroxycitronellal decreased. The non-reactive compounds α -isomethyl ionone, benzyl alcohol, amyl cinnamal and farnesol became reactive after photo-oxidative degradation. Overall, forced degradation resulted in four non-reactive fragrance compounds to display *in chemico* thiol reactivity, while ten out of 24 compounds remained inactive. Chemical degradation does not necessarily occur with generation of reactive species. Non-chemical activation may be involved for the 10 stable unreactive compounds.

1. Introduction

Contact with fragrance ingredients is one of the most common sources of adverse reactions such as allergic contact dermatitis (ACD). Due to their widespread use in cosmetic, toiletry and personal care products, avoiding exposure to fragrance ingredients is impractical. In such scenarios, identification of the source of a particular hazard can be a daunting task as some compounds are ubiquitously present in a plethora of products, typically as complex mixtures/formulations.

The bulk of evidence regarding the frequency of contact allergy to fragrance ingredients primarily relies on clinical data from patients with a history of allergic reactions or suspected ACD (Schnuch et al., 2007; Van Oosten et al., 2009). Based on such large population data, a list of commonly occurring constituents has been prioritized based on frequently recognized allergens (Larsen, 1977; Frosch et al., 2005). National and international surveys identified 26 fragrance ingredients

(24 pure compounds and 2 natural extracts) of potential concern due to a correlation between their occurrence in cosmetic products and the reported adverse effects. Such clinical trials usually require substantial efforts in planning and coordination at a multi-center level, as well as an extensive pool of patients to achieve statistical significance. Moreover, these studies are usually performed at a post-marketing stage on patients known for (or suspected of) a history of skin allergies.

The development and adoption of alternative methods is thus fundamental in order to minimize the use of human subjects as well as animals and to prevent harmful exposure of the consumer to potentially hazardous compounds. *In vivo* animal studies have been the method of choice for decades in skin sensitization risk assessment. As regulatory agencies are moving toward the replacement of animal studies, an impressive number of *in vitro*, *in chemico* and *in silico* methods have been developed and this list is constantly growing (Urbisch et al., 2015).

Skin sensitizers are typically small, lipophilic molecules with

Abbreviations: ACD, Allergic Contact Dermatitis; ACN, Acetonitrile; AOP, Adverse Outcome Pathway; DCYA, Dansyl Cysteamine; GC–MS, Gas Chromatography–Mass Spectrometry; GMP, Good Manufacture Practice; IFRA, International Fragrance Association; HTS, High Throughput Screening; MIE, Molecular Initiating Event; POV, Peroxide Value; RI, Reactivity Index

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electrophilic properties. The electrophilicity of a sensitizer is considered a key feature in the Molecular Initiating Event (MIE). The majority of skin sensitizers contain clearly identifiable reactive groups, which have been classified in six mechanistic domains (Aptula et al., 2005). Based on such unique chemical requirements, numerous *in chemico* and *in silico* methods have been proposed to classify potential sensitizers based on their ability to react with model nucleophiles (Ezendam et al., 2016). Interestingly, 13 of the 24 pure compounds identified as being the most recurrent fragrance allergens do not contain potentially reactive mechanistic domains. It has been hypothesized that some kind of chemical or biological activation may be involved in the generation of the reactive species (Karlberg et al., 2008). Compounds that can convert to sensitizers after chemical transformation are classified as pre-haptens, while pro-haptens are activated *in vivo* by xenobiotic mechanisms (Christensson et al., 2016). This distinction comes with consequences: in the case of pre-haptens the risk is intrinsic in the nature of the compound and could be minimized, for example by storing under proper conditions, addition of stabilizer(s) or appropriate formulation. In the case of pro-haptens, the compound may be chemically stable but the sensitization potential may be linked to bio-activation to reactive intermediates, genetic and/or individual predisposition. To complicate things further, some compounds may act as both pre- and pro-haptens.

Data obtained from individual case studies confirmed that chemical degradation has been linked to increased sensitization potency in animal experiments (Matura et al., 2002; Matura et al., 2003; Sköld et al., 2004; Hagvall et al., 2007; Christensson et al., 2008; Niklasson et al., 2013). Nonetheless, a systematic investigation of such correlation using non-animal methods is lacking. As a continuation of our ongoing investigation of the applicability of *in chemico* methods for skin sensitization potential of (un)known chemicals, the current study was undertaken to investigate the chemical reactivity of the 24 substances of concern using a recently developed high throughput method “HTS-DCYA” (Avonto et al., 2015). In addition, the correlation between their chemical stability (as well as peroxide accumulation) and changes in reactivity under forced degradation conditions was investigated. In order to establish purity and stability of the test articles, a GC–MS method for the analysis of the 24 compounds has also been developed. The remaining two natural extracts are complex mixtures and were therefore not included in the present investigation.

2. Material and methods

2.1. Chemicals and reagents

The list of chemicals examined in this study is presented in Tables 1 and 2. Lilial was purchased from Tokyo Chemical Industry. Lyrall was purchased from Chemtek Inc. The remaining fragrance ingredients, quinine monohydrochloride dehydrate (CAS 6119-47-7) and Quantofix® peroxides test sticks (0–25 mg/L) were purchased from Sigma-

Table 1
Most frequently reported fragrance allergens.

	Compound	CAS no.
1	Amyl cinnamal (<i>cis/trans</i>)	122-40-7
2	Amyl cinnamyl alcohol	101-85-9
3	Benzyl alcohol	100-51-6
4	Benzyl salicylate	118-58-1
5	Cinnamyl alcohol	104-54-1
6	Cinnamal	104-55-2
7	Citral	5392-40-5
8	Coumarin	91-64-5
9	Eugenol	97-53-0
10	Geraniol	106-24-1
11	(±)-Hydroxycitronellal	107-75-5
12	(±)-Lyrall (mixture of isomers)	31906-04-4
13	Isoeugenol (<i>cis/trans</i>)	97-54-1

Table 2
Less frequently reported fragrances allergens.

	Compound	CAS no.
14	Anisyl alcohol	105-13-5
15	Benzyl benzoate	120-51-4
16	Benzyl cinnamate	103-41-3
17	(±)-Citronellol	106-22-9
18	Farnesol	4602-84-0
19	α-Hexyl cinnamal (<i>cis/trans</i>)	101-86-0
20	(±)-Lilial	80-54-6
21	(R)-(+)-Limonene	5989-27-5
22	(R)-(–)-Linalool	78-70-6
23	Methyl 2-octynoate	111-12-6
24	(±)-α-Isomethyl ionone	127-51-5

Aldrich (St. Louis, MO, USA). The fluorescent compounds DCYA disulfide and DCYA were synthesized as described previously (Chittiboyina et al., 2015). Maleimide polymer-supported (SiliaBond® Maleimide, ≥ 0.64 mmol/g) was purchased from SiliCycle (Quebec City, Quebec, Canada). Standardized buffer solution pH 10, microcentrifuge tubes, polypropylene solvent-resistant 96-well microplates and 1.5 mL methacrylate cuvettes were purchased from Fisher Scientific (Suwanee, GA, USA).

2.2. Instrumentation and data analysis

The fluorescent readings were acquired on a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, California, USA). Data were acquired and processed using SoftMax Pro 5 (Molecular Devices, Sunnyvale, California, USA) and Microsoft Excel 2013 software.

2.3. GC–MS analyses

The GC–MS system consisted of an Agilent 7890 gas chromatograph and an Agilent 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The injections were made with an Agilent 7693 autosampler. The data were collected using ChemStation software (Version E.02.02). An Agilent DB-5MS GC capillary column (30 m, 0.25 mm, 0.25 μm) with (5%-phenyl)-methylpolysiloxane as the stationary phase was used for the analysis. The instrument was operated at a constant flow rate with helium as the carrier gas at 1 mL/min. The inlet temperature was 250 °C, and the injector was operated in a split mode with a split ratio of 50:1. One microliter was injected. The oven temperature program started at an initial temperature of 60 °C (held for 1 min), then the oven was ramped up to 280 °C at 5 °C/min, and held at 280 °C for 5 min. The mass selective detector was operated in scan mode with the mass range from *m/z* 40 to 500.

2.4. Sample preparation for stability studies by GC–MS

At the specific sampling day, about 5 mg of compounds were accurately weighed and dissolved in acetonitrile to a final concentration of 5 mg/mL. Two hundred microliters of sample were then diluted to 1 mg/mL in acetone containing 1 mg/mL of internal standard (tetradecane). Each vial was analyzed in duplicate (2 vials/compound). The initial stock solution in acetonitrile (5 mg/mL) was also used for HTS-DCYA assays.

2.5. Forced degradation studies

About 1 g of each compound (in duplicates) was placed in a 1 dram glass vial closed with a Teflon septum, constantly mixed using a magnetic stirrer and irradiated for 6 h light/dark cycles using a light source simulating daylight exposure conditions. For irradiation purposes, cool daylight compact fluorescence light bulbs (860 lm, 6500 K, 60 W

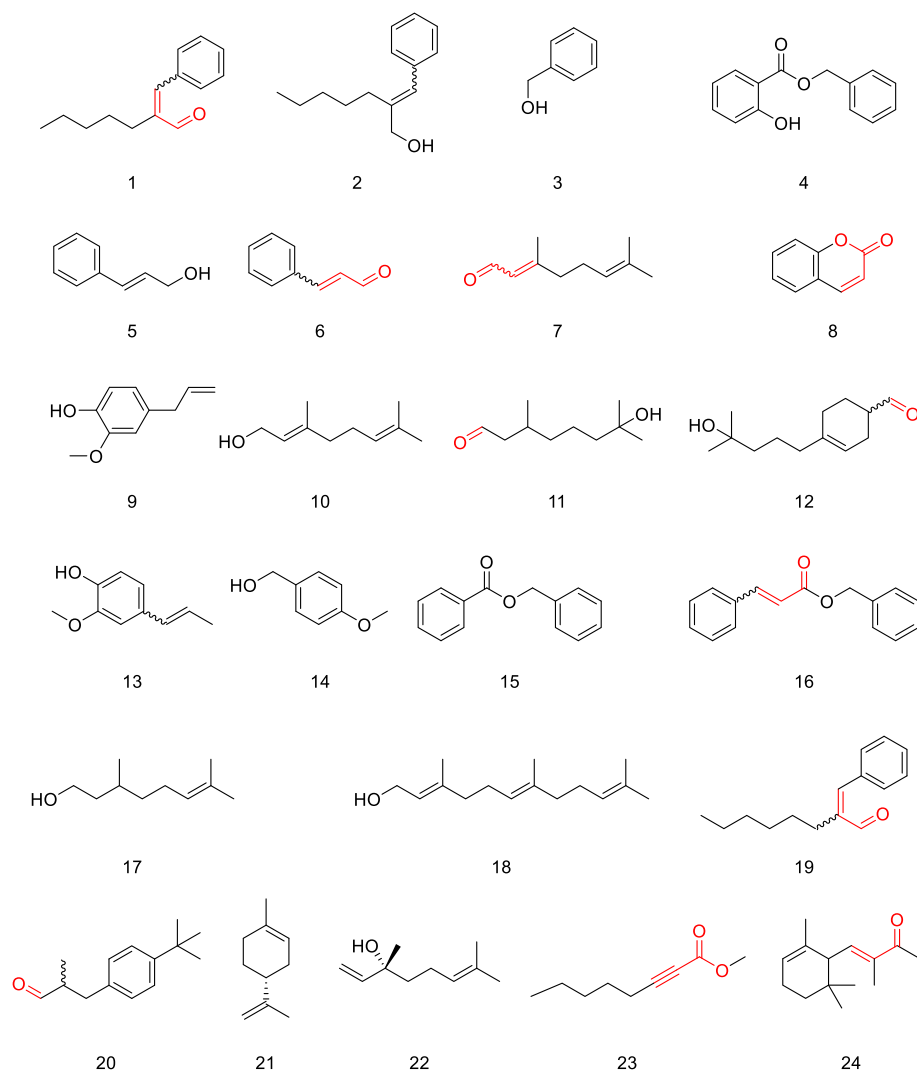


Fig. 1. Chemical structures of the 24 compounds investigated. Structural alerts suggestive of chemical reactivity are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

equivalent, Philips®) were used. The samples were distributed in a radius around the light source at a sufficient distance to achieve a minimum intensity of about 1666 lx/h for each sample. The samples were irradiated for at least 100 days to meet the minimum exposure as required by ICH guidelines (ICH, 1998). An air pump was used to provide a constant air flow. At specific intervals, 50 mg of each sample was collected and stored at -20°C for reference. At the same time, about 5 mg of material were aliquoted for both GC–MS and HTS-DCYA experiments.

2.6. Actinometric assay

Irradiation conditions were validated using an actinometric assay to ensure that satisfactory light exposure was achieved during the stability study (ICH, 1996). Quinine monohydrochloride dihydrate (2% w/v in ultrapure water) was used for the actinometric assay. About 3.6 mL of quinine solution was placed in two vials identical to the one used for the test articles and irradiated along with the test articles (6 h dark/light cycles). Dark controls (DC) were obtained by placing the same quinine solution wrapped in aluminum foil alongside the irradiated control (IC). The absorbance of the irradiated controls and the dark control were evaluated spectrophotometrically by quantifying the UV absorbance at 400 nm using 1 cm path length cuvettes. The change in absorbance was calculated as $\Delta A = A(\text{IC}) - A(\text{DC})$. The minimum irradiation time, sufficient to obtain a change in absorbance of at least 0.9, was calculated to be 75 days.

2.7. Determination of peroxide content

The compounds were dissolved in ultrapure water to a concentration of 10 mg/mL. Eight microliter of such solution was then loaded on peroxide strips and the peroxide content was determined by colorimetric assay.

2.8. HTS-DCYA

The chemical reactivity was assessed using the *in chemico* HTS-DCYA method as previously described (Avonto et al., 2015). Briefly, a 2.5 mM solution of DCYA was mixed with an equal volume of 5.0 mM of test article, both prepared in acetonitrile. The resulting solution was then split in two microcentrifuge tubes (150 μL in each tube). Thirty microliters of aqueous pH 10 buffer was added to the reaction (R) tubes; 30 μL of acetonitrile was added to the second tube (blank, BI) in place of the buffer. DCYA controls were prepared by mixing the 2.5 mM solution of DCYA to an equal volume of acetonitrile. The obtained DCYA solution (150 μL) was then incubated with 30 μL of aqueous pH 10 buffer (negative control, NC), or with 30 μL of acetonitrile (positive control, PC). The samples were incubated for about 20 min at room temperature. Thirty mg of maleimide resin was then added, the suspension was vortexed and incubated for 90 min with vigorous shaking. The samples were centrifuged at 10,000 rpm (10 min), then 60 μL of the supernatant was diluted to a final volume of 1 mL with ACN. Two hundred μL (in triplicate) of the diluted solutions was put into a 96 well solvent-

resistant microplate. Each plate was read in triplicate using fluorescence end-point readings. End-point quantifications were performed by fluorescence HTS method (emission wavelength 520 nm, excitation 350 nm, cutoff 420 nm). The Reactivity Index (RI) was calculated based on the average of 9 readings per sample (3 readings/well in triplicate) as follows:

$$RI = 100 \times ((BI - R)/(PC - NC) - (PC - BI)/PC)$$

3. Results

As covalent binding to skin proteins is considered the first MIE in the Adverse Outcome Pathway (AOP) for skin sensitization, the 24 compounds of concern were tested *in chemico* to determine their ability to bind to the fluorescent thiol, dansyl cysteamine (DCYA), as a surrogate of the biological target. The compounds were then subjected to a forced degradation study to determine whether their chemical reactivity depended upon stability and generation of reactive species caused by prolonged exposure to air and light.

3.1. DCYA reactivity of 24 compounds

The list of 24 synthetic and natural fragrance ingredients of concern includes both aliphatic and aromatic compounds, with a prevalence of cinnamyl derivatives and terpenoids. The initial purity of each compound was assessed by GC–MS. Eleven compounds contain at least one structural alert for skin sensitization (Fig. 1). The compounds were tested for reactivity using the HTS-DCYA method, and the results are reported in Table 3. Only 5 out of 24 compounds resulted in clear DCYA depletion (6, 7, 12, 13, 23), while 6 compounds (9–11, 17, 21, 22) resulted in a borderline reactivity, in which the RI is close to cutoff threshold ($RI \geq 5$) with a high standard deviation. The reactivity threshold was based on previously established values (Avonto et al., 2015) and it cannot be used as a stand-alone index for potency classification. Six compounds with structural alerts (1, 8, 16, 19, 20 and 24) and seven compounds without structural alerts (2–5, 14, 15 and 18) were found to have minimal to no-reactivity. It appears that the observed reactivity with DCYA does not correlate with the presence of

structural alerts.

3.2. Chemical stability upon forced degradation

All the compounds were subjected to forced degradation by simultaneous exposure to air and light for 3 months. Qualitative and quantitative changes in the chemical composition were examined by GC–MS. The stability of the test articles varied greatly among the compounds: seven compounds were highly unstable (Fig. 2a and b), with significant loss ($> 10\%$) observed within 15 days and $> 50\%$ depletion at the end of the study. A second set of compounds was found moderately stable (Fig. 3a and b), resulting in $< 10\%$ depletion within 30 days, while the remaining eight compounds were found to be stable even after three months (Fig. 4a and b) under forced degradation conditions. Discordant results were obtained for citronellol, with significant discrepancies in quantification of duplicates (Fig. S1). Both duplicates tested positive for DCYA depletion (Fig. S2) and for the presence of peroxides (Table S1), but GC–MS quantification of one of the two replicate showed little, if any, variation. Based on reported literature (Rudbaeck et al., 2014) and on the stability of structurally related monoterpene alcohols, such as linalool and geraniol, citronellol can be considered unstable upon forced degradation, although no reliable comparative stability information could be obtained in the current study. Benzyl esters along with aromatic alcohols, eugenol, and methyl 2-octynoate were stable after three months. As anticipated, the aliphatic terpenoids were particularly unstable, leading to complex GC profiles with numerous peaks.

3.3. Reactivity of the 24 compounds after forced degradation

The increase in chemical reactivity upon forced degradation was investigated using the HTS-DCYA method, and the comparative results are summarized in Fig. 5. At the end of the forced degradation study, the degree of reactivity in terms of DCYA depletion was significantly higher for 10 of 24 compounds, with at least 20 RI value increase. Isoeugenol, α -isomethyl ionone, lylal and limonene reactivity increased over 50 RI unit after three months, followed by citronellol, geraniol, linalool, benzyl alcohol, amyl cinnamal and farnesol. Seven of the

Table 3
Chemical reactivity of the 24 fragrance ingredients.

N.	Name	(HTS-DCYA) RI	St. dev.	Structural alert	Classification ^a
1	Amyl cinnamal (<i>cis/trans</i>)	4.4	0.3	YES	0
2	Amyl cinnamyl alcohol	2.6	0.7	NO	0
3	Benzyl alcohol	4.1	1.7	NO	0
4	Benzyl salicylate	2.0	0.2	NO	0
5	Cinnamyl alcohol	2.8	3.8	NO	0
6	Cinnamal	12.8	3.0	YES	1
7	Citral	16.3	4.3	YES	1
8	Coumarin	5.1	3.1	YES	0
9	Eugenol	8.4	4.3	NO	1
10	Geraniol	7.7	2.0	NO	1
11	($\pm$)-Hydroxycitronellal	7.2	1.7	YES	1
12	($\pm$)-Lylal (mixture of isomers)	15.2	0.04	YES	1
13	Isoeugenol (<i>cis/trans</i>)	13.4	2.1	NO	1
14	Anisyl alcohol	2.5	0.7	NO	0
15	Benzyl benzoate	1.1	0.8	NO	0
16	Benzyl cinnamate	5.6	0.9	YES	0
17	($\pm$)-Citronellol	6.8	1.9	NO	1
18	Farnesol	5.0	4.0	NO	0
19	α -Hexyl cinnamal (<i>cis/trans</i>)	4.8	0.4	YES	0
20	($\pm$)-Lilial	1.8	3.1	YES	0
21	(R)-(+)-Limonene	8.6	5.3	NO	1
22	(R)-(-)-Linalool	6.9	4.0	NO	1
23	Methyl 2-octynoate	54.2	4.1	YES	1
24	($\pm$)- α -Isomethyl ionone	− 0.9	3.8	YES	0
	Total			11	11

^a 0 = negative 1 = positive.

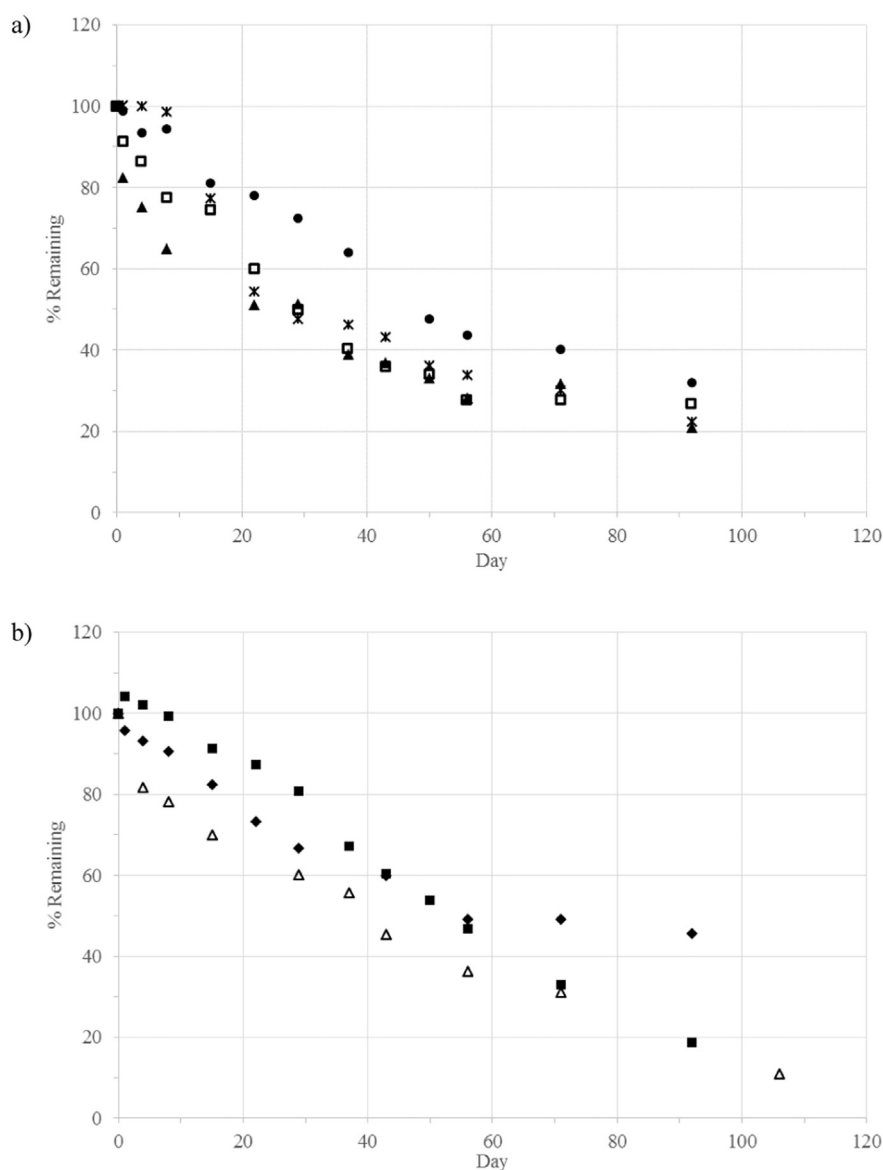


Fig. 2. Highly unstable compounds under forced degradation conditions (quantified by GC-MS): a) ● = Cinnamal; □ = Citral; ✕ = Geraniol; ▲ = Hydroxycitronellal; b) ◆ = Farnesol; Δ = Lilial; ■ = Limonene.

compounds were classified as moderately stable by GC-MS quantification, while three (geraniol, farnesol and limonene) were highly unstable.

For two aldehydes, hexyl cinnamal and hydroxy citronellal, the degradation was not accompanied by an increase in RI. Degradation of hydroxycitronellal was accompanied by formation of hydroxycitronellallic acid (Fig. S3) without reactivity increase. Similarly, the oxidation process of cinnamal leads to formation of cinnamic acid, whereas no significant variation in reactivity of cinnamal over time was observed.

3.4. Determination of peroxide value (POV)

Organic and inorganic peroxides are frequently generated as reactive intermediates during oxidation processes, thus the peroxide contents before and after aging were also evaluated. About 67% of the compounds showed an increased concentration of POV after three months, with the highest peroxide value assessed for benzyl alcohol (Table S1).

The POV was in all cases estimated to be lower than the interference threshold previously established for the HTS-DCYA method (Avonto et al., 2016), therefore unlikely to contribute to increased reactivity as a result of peroxide-induced auto-oxidation of DCYA. Moreover, no clear

association between the POV and reactivity was found. These results are in agreement with our previous findings: an increase in peroxide content may be regarded as a clue of ongoing oxidation processes, but not necessarily as an indicator of increased thio-reactivity with *in chemico* assays. It is nonetheless worth noting that decomposition of unstable terpenoids led to accumulation of peroxides and reactive species.

4. Discussion

The results obtained from forced degradation and reactivity studies offer some interesting insights. A few examples are listed below to highlight the importance of chemical stability and implications to chemical reactivity.

4.1. Eugenol and isoeugenol

Eugenol and isoeugenol are two deceptively similar compounds. The sensitization mechanisms of eugenol and isoeugenol have been the object of various studies. Both clinical and mechanistic evidence led to the conclusion that two different activation pathways are associated with the skin sensitization potential of these two compounds (Barratt and Basketter, 1992). Bertrand and co-workers' structure-activity

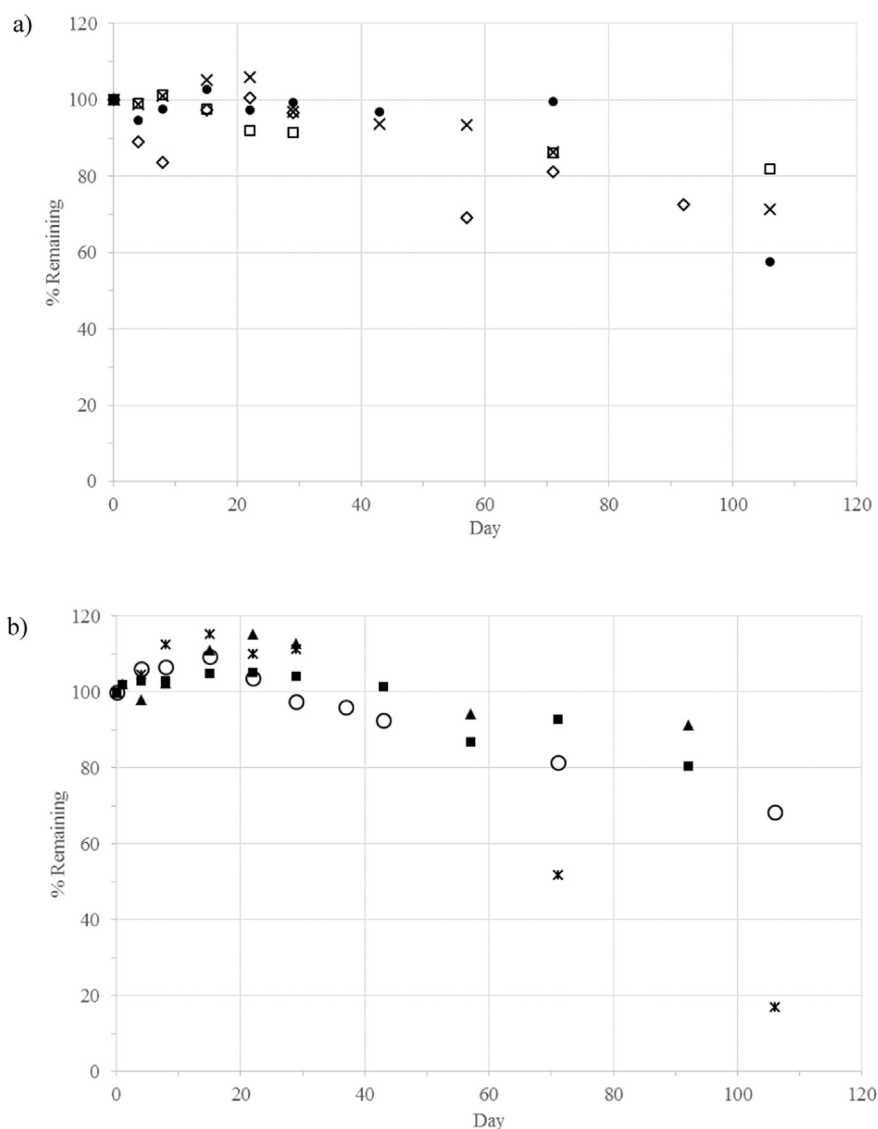


Fig. 3. Moderately unstable compounds under forced degradation conditions (quantified by GC–MS): a) • = Amyl cinnamal; □ = Benzyl alcohol; ◇ = Cinnamyl alcohol; × = Lyrar; b) ▲ = Isoeugenol; ✕ = Hexyl cinnamal; ■ = Linalool; ○ = α-Isomethyl ionone.

correlations led to the conclusion that enzymatic demethylation followed by oxidation to *o*-quinone may be involved in the hapten generation from eugenol, while isoeugenol activation may undergo direct photo-oxidation to *p*-quinone methide (Bertrand et al., 1997). Based on the result discussed herein, eugenol was found to be more stable and less reactive under oxidative conditions, while the reactivity index of isoeugenol increased over 50 RI units. These findings corroborate the hypothesis that radical oxidation (whether enzymatic or non-enzymatic) of isoeugenol may lead to the generation of reactive species, while eugenol oxidation may be primarily mediated by bioactivation.

4.2. Cinnamal and cinnamyl alcohol

From GC–MS results, cinnamal was found to oxidize readily to its acid derivative as a major identifiable degradation product (Fig. S4). Under the same conditions, cinnamyl alcohol degradation was slower (about 27% degradation versus 68% of cinnamal), and a peak corresponding to cinnamal (16.71 min, Fig. S5) was observed as one of the minor degradation by-products. The amount of aldehyde formed was nonetheless insufficient to cause a major increase in reactivity. Niklasson and co-workers previously reported a 15 days half-life for cinnamyl alcohol, depending on the oxidation conditions, when tested as an alcoholic solution (Niklasson et al., 2013). In their experiments,

the auto-oxidized mixture was found to contain cinnamal along with cinnamic acid and highly sensitizing epoxy derivatives (Niklasson et al., 2014). Cinnamal and cinnamyl alcohol are considered cross-sensitizers due to metabolic oxidation.

4.3. Amyl cinnamal, hexyl cinnamal and amyl cinnamyl alcohol

Similarly to the cinnamal and cinnamyl alcohol, a preferential stability was found for the alcohol over the aldehyde counterparts. About 42 and 83% losses were quantified for amyl cinnamal and hexyl cinnamal, respectively, while no loss of amyl cinnamyl alcohol was observed at the end of the forced degradation period. Degradation of amyl cinnamal was accompanied by a seven fold increase in reactivity while amyl cinnamyl alcohol was found to be non-reactive.

5. Conclusions

In the present work, 24 fragrance ingredients of concern were investigated to determine how chemical stability affects the DCYA-reactivity by using a novel fluorescence-based *in chemico* technique. At the end of the forced degradation study, 8 of 13 compounds from the list of most common allergens and 6 of 11 from the list of less common ones were found to be reactive from the *in chemico* HTS-DCYA results.

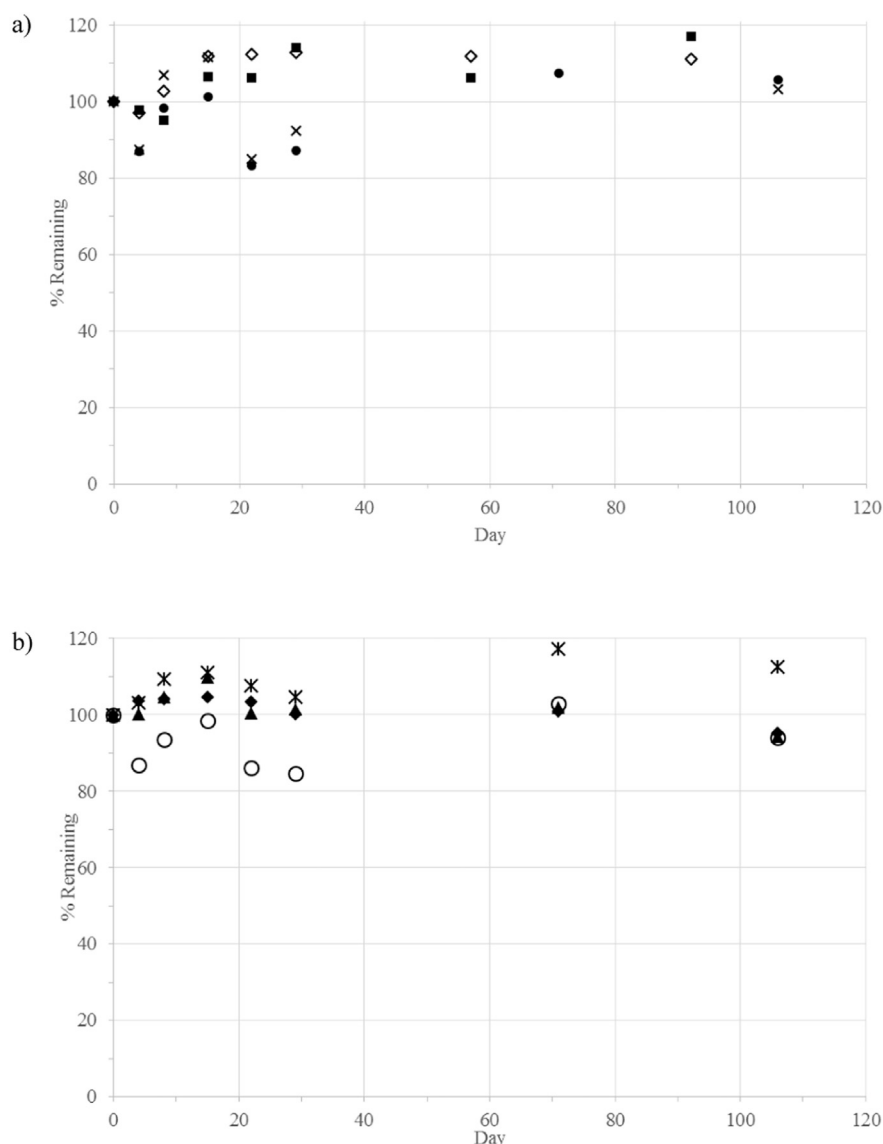


Fig. 4. Compounds stable under forced degradation conditions (quantified by GC-MS): a) • = Amyl cinnamyl alcohol; × = Benzyl salicylate; ◇ = Coumarin; ■ = Eugenol; b) ○ = Anisyl alcohol; ▲ = Benzyl benzoate; ✕ = Benzyl cinnamate; ◆ = Methyl 2-octynoate.

As a general observation, terpenes, terpinene alcohols and aldehydes, followed by aromatic aldehydes and alcohols, were found to be the most susceptible to oxidation. The susceptibility of terpenoids to chemical oxidation is well known (Blakeway et al., 1987) and has been the object of intense studies by flavour and fragrance chemists (Bernhard and Marr, 1960; Pokorný et al., 1998; Hagvall et al., 2007; Turek and Stintzing, 2013).

Aromatic esters and the acetylenic derivative methyl 2-octynoate were quite stable and minimal changes in reactivity were found, although some of these compounds were found to have increased POVs upon aging. The stability of methyl 2-octynoate was in contrast with the behavior of a previously investigated natural alkyne, tonghaosu, which rapidly converted into a highly reactive degradation by-product upon photo-oxidation. This was not unexpected as the stability and reactivity of triple bond strictly depends on the nature of the substituents as well as extended conjugation to the alkyne moiety (Avonto et al., 2017).

In the majority of cases, degradation of a chemical was not accompanied by a proportionate increase of a singular by-product, but rather by the formation of mixtures containing various oxidized by-products. Even in the case of cinnamal and hydroxycitronellal, where specific by-products were found, the amount of product formed did not account for 100% of the degraded parent compound. Multiple factors could be involved in the apparent “loss” of product: 1) generation of

non-volatile derivatives that are non-detectable with the applicable analytical conditions (including high molecular weight dimers or non-volatile oxidized by-products); 2) formation of volatile but thermally unstable compounds which may decompose under GC-conditions (e.g. peroxides, radical species) and 3) presence of equilibria where the degradation by-products may convert back to the original parent compound.

Chemical instability did not necessarily translate in increased thio-reactivity, therefore a case-by-case approach is recommended. All the compounds with borderline reactivity (limonene, linalool, citronellol and eugenol) were more reactive after forced degradation. Since none of these compounds contain a reactive mechanistic domain, it is plausible that the observed reactivity at day zero may be related to oxidation by-products of these compounds. The presence of minor or undetected reactive impurities in the fresh samples cannot be excluded and may be responsible for the observed initial reactivity. Another explanation could be the occurrence of *in situ* activation in the experimental reaction conditions. It is nonetheless probable that chemical oxidation of these compounds plays an important role in the generation of reactive compounds. Four non-reactive compounds (α -isomethyl ionone, benzyl alcohol, amyl cinnamal and farnesol) were found to cause DCYA depletion after forced degradation, while for six other compounds (geraniol, limonene, linalool, lylal, isoeugenol and

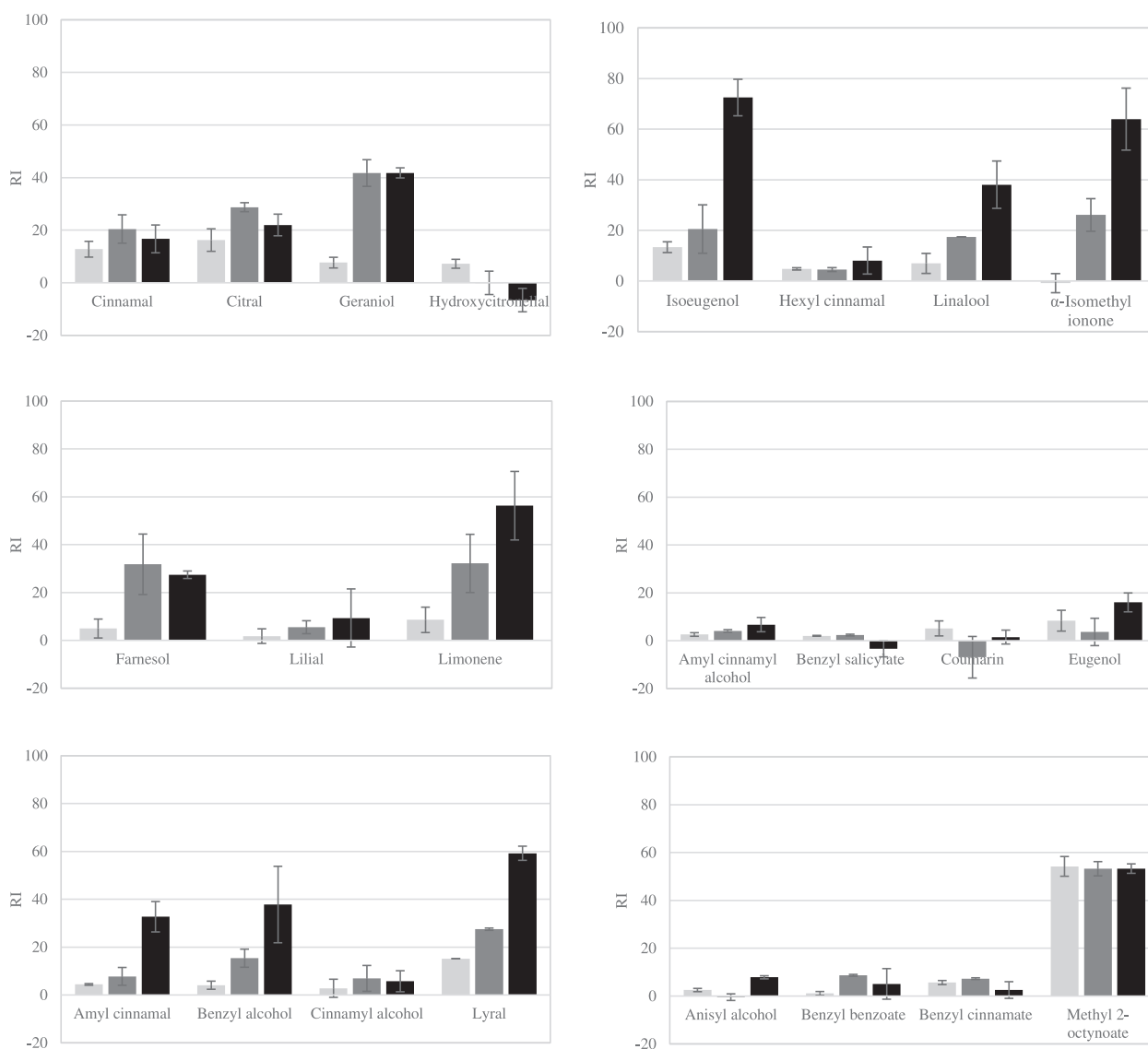


Fig. 5. Chemical reactivity of fragrance ingredients in the HTS-DCYA assay: ■ = Day 0; ■ = Day 29; ■ = Day 92. Citronellol results are reported in Supplemental material.

citronellol) the binary classification remained unchanged although a remarkable change in degree of reactivity was observed. The initial purity as well as storage conditions are thus extremely important for hazard assessment of these ingredients. It is also worth remembering that the compounds were tested in accelerated conditions, to explore for potential hazards in a reasonable time frame. These circumstances do not reflect the typical storage and formulations conditions to which these compounds are normally subjected, because preservatives and stabilizing agents are typically included in personal care product formulations to extend the shelf life. A study on commercial products manufactured under GMP guidance demonstrated how following International Fragrance Association (IFRA) standards typically results in products which comply with safety criteria with regard to potential auto-oxidation processes (Api et al., 2015). In any case, the data obtained herein are very important to raise awareness of potential concerns, to determine the safety measures in occupational hazard risk assessment, proper storage and handling conditions and to gain insight about the generation of potentially reactive intermediates and their correlation with skin sensitization adverse events.

It remains to be established how nine fragrance compounds that remained DCYA-unreactive upon forced degradation (#2, 4, 5, 8, 14, 15, 16, 19 and 20) exert skin sensitization properties. One possibility is

that *in chemico* reactivity does not accurately mimic conditions in intracellular milieu. With respect to that, seven (#4, 5, 8, 15, 16, 19 and 20) of these nine chemicals were tested in Keratinosens™ and h-CLAT cellular assays. All were found positive in the Keratinosens™ assay, with one exception (Urbisch et al., 2015). Thus, a clear-cut support for this explanation is at present not available. Another possibility is that metabolic conversion occurs *in vivo* and that (some of) these nine compounds are in effect pro-haptens. This issue has been examined for the same seven compounds and it was found that only cinnamyl alcohol displays features of pro-hapten (Patlewicz et al., 2016). Activation of keratinocytes and dendritic cells mediated pathways caused by the oxidized chemicals is currently under investigation. These results, combined with the results herein obtained, could provide useful information on the three major key events and thus provide a more comprehensive overview on how oxidation of the 24 chemicals may affect their sensitization potential. Finally, there is also a possibility that some of these chemicals may be utilizing hapten-independent mechanisms of skin sensitization (Vukmanović and Sadrieh, 2017). For example, reactivity to proteins is not required for binding to HLA molecules, which occurs mostly thanks to hydrogen bond formation. Few examples of drugs or metals binding to HLA exist, but it remains to be explored for fragrances.

Overall, the present study showed how chemical stability is a critical factor to be considered for fragrance ingredients. Instability may lead to the generation of species of toxicological concern and thus should be taken into consideration in the development of safe formulations. For some of the compounds, lack of reactivity as well as stability indicate that other activation pathways or biological targets may be associated with establishing the skin sensitization potential.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The <http://dx.doi.org/10.1016/j.tiv.2017.09.007> associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplemental material includes chemical stability and reactivity data of citronellol, estimation of peroxides value before and after forced degradation, GC–MS chromatograms of hydroxycitronellal, cinnamal and cinnamyl alcohol before and after forced degradation. Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.tiv.2017.09.007>.

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Chemical stability and *in chemico* reactivity of 24 fragrance ingredients of concern for skin sensitization risk assessment.

Supplementary material

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Figure S1. Stability of citronellol upon forced degradation.

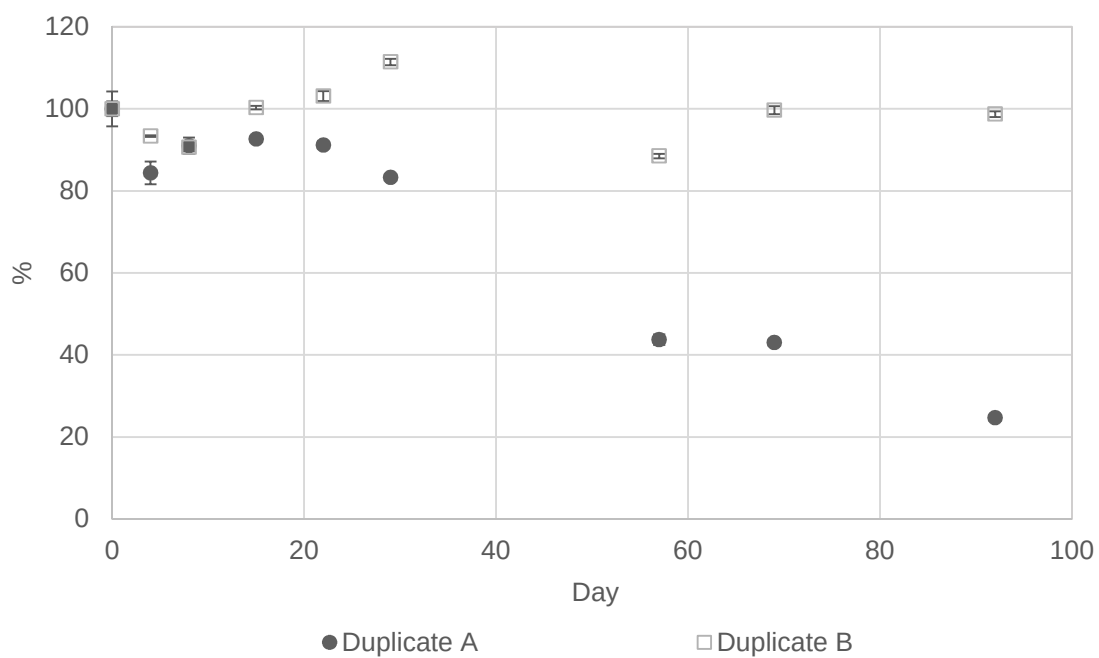


Figure S2. *In chemico* HTS-DCYA reactivity of citronellol in HTS-DCYA assay.

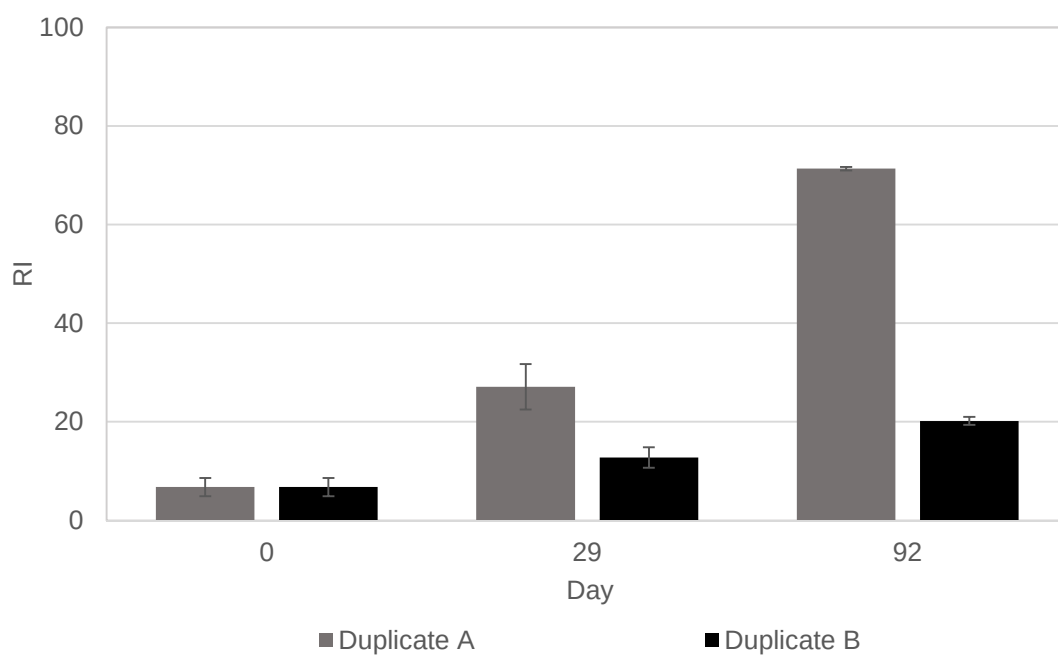


Table S1. Summary of degradation study of the 24 fragrance ingredients.

N.	Name	Structural alert	HTS-DCYA (RI)		GC-MS (% Remaining)			POV (mg/L)	
			Before	After	1 mo.	3 mo.	Stability	Before	After
1	Amyl cinnamal (<i>cis/trans</i>)	Michael acceptor/Schiff Base	4.4 ± 0.3	32.7 ± 6.4	99.5	57.5	Moderate	0	0.3-2
2	Amylcinnamyl alcohol	Pro-Michael acceptor/Schiff Base	2.6 ± 0.7	6.7 ± 3.0	87.3	105.8	Stable	0	10-11
3	Benzyl alcohol	Pro-Michael acceptor	4.1 ± 1.7	37.8 ± 16.0	91.5	81.8	Moderate	3	40
4	Benzyl salicylate		2.0 ± 0.2	-3.4 ± 3.4	92.3	103.4	Stable	0	0-0.2
5	Cinnamyl alcohol	Pro-Michael acceptor/Schiff Base	2.8 ± 3.8	5.7 ± 4.4	96.7	72.6	Moderate	0	0.5
6	Cinnamal	Michael acceptor/Schiff Base	12.8 ± 3.0	16.7 ± 5.3	72.5	32.0	Unstable	2	0.3-8
7	Citral	Michael acceptor/Schiff Base	16.3 ± 4.3	22.0 ± 4.1	49.8	26.6	Unstable	1	0.5-3
8	Coumarin	Michael acceptor	5.1 ± 3.1	1.5 ± 2.9	112.9	111.1	Stable	0	0
9	Eugenol	Pro-Michael acceptor	8.4 ± 4.3	16.0 ± 3.9	114.2	117.0	Stable	0	0
10	Geraniol	Pro-Michael acceptor/Schiff Base	7.7 ± 2.0	41.8 ± 1.9	47.8	22.5	Unstable	3	9-30
11	(±)-Hydroxycitronellal	Schiff Base	7.2 ± 1.7	-6.6 ± 4.4	51.3	20.9	Unstable	0.6	1-2
12	(±)-Lylal (mixture of isomers)	Schiff Base	15.2 ± 0.04	59.2 ± 3.0	96.8	71.3	Moderate	0	0
13	Isoeugenol (<i>cis/trans</i>)	Pro-Michael acceptor	13.4 ± 2.1	72.5 ± 7.2	112.9	91.3	Moderate	0	0
14	Anisyl alcohol	Pro-Michael acceptor/SNAr	2.5 ± 0.7	7.8 ± 0.6	84.5	93.9	Stable	0.5	15
15	Benzyl benzoate		1.1 ± 0.8	5.1 ± 6.4	101.4	94.2	Stable	0	7-10
16	Benzyl cinnamate	Michael acceptor	5.6 ± 0.9	2.5 ± 3.5	104.6	112.6	Stable	0	0
17	(±)-Citronellol	Pro-Schiff base	6.8 ± 1.9	45.8 ± 25.6	97.4	61.7	Moderate	0.5	15-20
18	Farnesol	Pro-Michael acceptor/Schiff Base	5.0 ± 4.0	27.4 ± 1.6	66.7	45.5	Unstable	1	3-9
19	α-Hexyl cinnamal (<i>cis/trans</i>)	Michael acceptor/Schiff Base	4.8 ± 0.4	8.1 ± 5.3	111.4	16.9	Moderate	0	0.3-0.5
20	(±)-Lilial	Schiff Base	1.8 ± 3.1	9.4 ± 12.1	60.2	10.9	Unstable	0	0.2-1
21	(R)-(+)-Limonene	None	8.6 ± 5.3	56.3 ± 14.3	80.8	18.7	Unstable	1	5-12
22	(R)-(-)-Linalool	None	6.9 ± 4.0	38.0 ± 18.5	104.2	80.4	Moderate	0.5	11-20
23	Methyl 2-octynoate	Michael acceptor	54.2 ± 4.1	53.3 ± 2.0	100.1	95.2	Stable	0.5	10-11
24	(±)-α-Isomethyl ionone	Michael acceptor	-0.9 ± 3.8	63.9 ± 12.2	97.6	68.3	Moderate	0	10-12
Positive		11	11	14				11	19
Unstable (Moderate-High)					16				

Figure S3. GC-MS chromatogram of hydroxycitronellal before and after forced degradation.

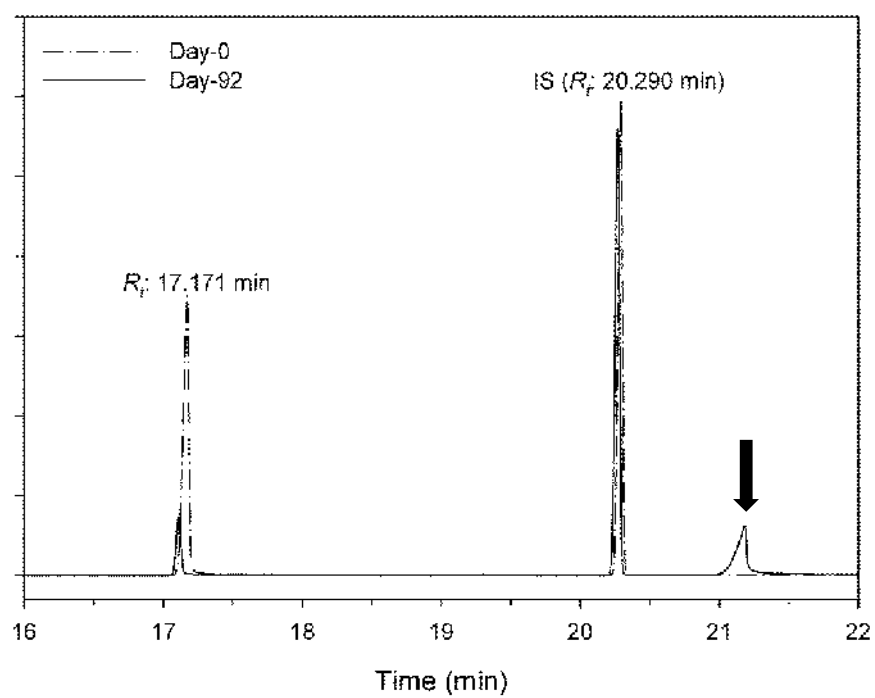


Figure S4. GC-MS chromatogram of cinnamal before and after forced degradation.

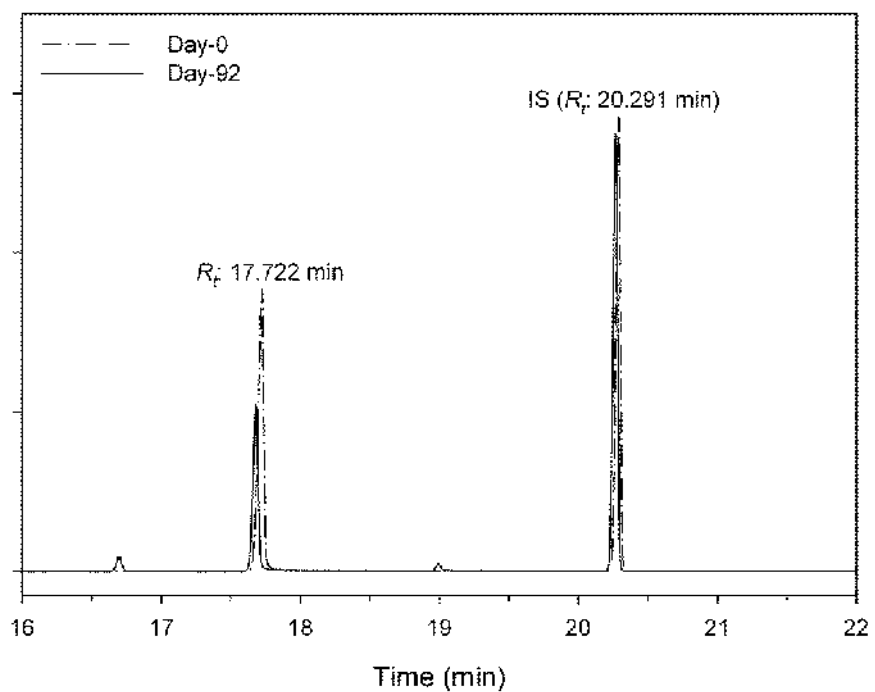
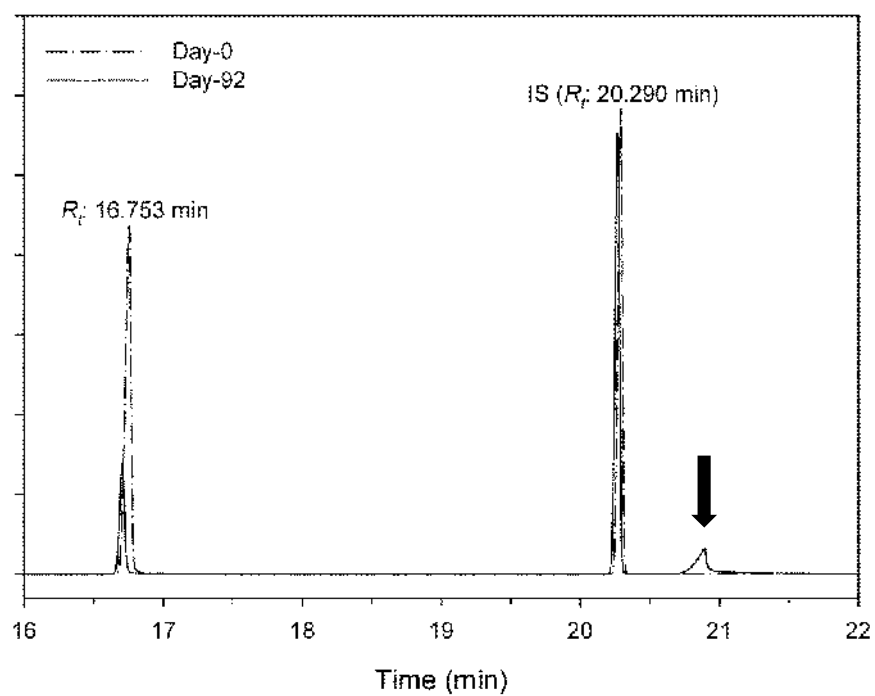


Figure S5. GC-MS chromatogram of cinnamyl alcohol before and after forced degradation.

