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ORIGINAL ARTICLE

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Allergic reactivity for different dilutions of eugenol in repeated open application test and patch testing

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Abstract

Background: Eugenol is a known contact sensitiser included in fragrance mix I.

Objective: To assess the allergic reactivity to eugenol in different concentrations using patch test as well as repeated open application test (ROAT).

Methods: Overall 67 subjects from 6 European dermatology clinics participated in the study. The ROAT was performed for 21 days twice a day, applying 3 dilutions of eugenol (2.7%–0.5%) and a control. Before and after the ROAT, patch testing with 17 dilutions of eugenol (2.0%–0.00006%) and controls was performed.

Results: Out of the 34 subjects with contact allergy to eugenol, 21 (61.8%) showed a positive patch test before ROAT was performed, the lowest positive concentration was 0.031%. The ROAT was positive in 19 (55.9%) of the 34 subjects, the time until a positive reaction occurred was negatively associated with the concentration of the ROAT solution, as well as with the allergic reactivity of the subjects as defined by patch testing. In the patch test after ROAT, 20 of the 34 test subjects (58.8%) showed a positive reaction. In 13 (38.2%) of the 34 test subjects, the patch test result was not reproduceable, still 4 (31.0%) of these 13 subjects developed a positive ROAT.

Conclusion: Eugenol can provoke a positive patch test reaction in a very low dose; besides, this hypersensitivity may persist even if a former positive patch test is not reproduceable.

KEYWORDS
allergic contact dermatitis, contact allergy, delayed hypersensitivity, elicitation threshold, eugenol, ROAT

1 | INTRODUCTION

Eugenol is a member of the phenylpropanoids class of chemical compounds. It is a clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil, nutmeg, cinnamon, basil and bay leaf. Eugenol is used in perfumes, flavourings, essential oils and in medicine as a local antiseptic and local anaesthetic. Zinc oxide eugenol is used in bonding materials in dentistry. Eugenol is a contact

sensitiser[1] included in fragrance mix I, a mixture of common perfume allergens, used to detect contact allergy in the European baseline series of contact allergens.

It is known that not all patients with a positive patch test to a specific substance (e.g., fragrance materials) develop allergic contact dermatitis when the substance is applied in a use test like the repeated open application test (ROAT).[2,3] During ROAT, a dilution containing a specific substance is applied without occlusion to a predefined skin area of the patient. The ROAT is intended to simulate normal contact with the substance in daily life (e.g., when using scented products).[3,4] A negative ROAT on healthy skin may become positive in the same patient when applied to damaged skin.[3,5] In addition, the concentration of the substance of interest in the ROAT dilution, as well as the skin site where the ROAT is applied, can play a role in the elicitation of allergic contact dermatitis.[6] It has also been shown that for some substances, inter individual patch test reactivity affects the elicitation process.[5]

The objective of this study was (a) to determine the threshold for elicitation of contact allergy to eugenol in patch testing, (b) to determine whether the initial patch testing and ROAT would affect subsequent elicitation thresholds and (c) to investigate whether there is an association between the degree of eugenol hypersensitivity and the outcome of the ROAT by comparing patch test reactivity with the ROAT concentration needed to exhibit a positive reaction and/or the duration of ROAT needed to develop a positive ROAT.

2 | MATERIALS AND METHODS

2.1 | Study design

The study was performed between 2013 and 2018 and was conducted in two phases, adapted in part from Andersen et al.[6] and Johansen et al.[7] In Phase I, a patch test and a use test (ROAT) were started concomitantly (Figure 1). The end of the ROAT period was followed by a 4-week rest period and then Phase II, which consisted of a patch test only. The study was performed in a double-blind fashion: two dermatologists were involved in reading the results of each subject, Dermatologist A read the patch test results and Dermatologist B read the ROAT results.

2.2 | Subjects

Overall 67 subjects were recruited into this multi-centre study conducted in 6 European dermatological clinics. All subjects were normal healthy men or women without any active eczema, aged ≥18 years and able to give informed consent as well as to follow all study procedures. Out of the 67 subjects a total of 34 subjects (test subjects) had a positive patch test to eugenol in the previous 10 years. The test subjects had a mean age of 47.1 years (SD 15.7) and 16 (47.1%) were female. The 33 control subjects were also recruited out of the patients of the referring clinics, their mean age was 48.1 years (SD 15.1) and 25 (75.8%) were female. The control subjects had a history of allergic contact dermatitis but without hypersensitivity to fragrance materials, including eugenol, balsam of Peru and/or fragrance mixtures (Table 1). All subjects had previously been referred to the participating clinics for diagnosis and treatment and the positive test has been conducted in the participating clinic.

2.3 | Test preparations

Eugenol from the same batch was used for all test solutions (patch test and ROAT). All samples of eugenol were kept refrigerated and protected from light. Eugenol was supplied by the Research Institute for Fragrance Materials (RIFM, Woodcliff Lake, New Jersey, USA) as a pure formulation without any additives (e.g., antioxidants). Diethylphthalate (DEP) is in combination with ethanol, a common vehicle in scented hydro-alcoholic products. It is thus close to what consumers are exposed to and was therefore used in the test preparations. DEP was purchased from Sigma Aldrich, Steinheim, Germany and ethanol from

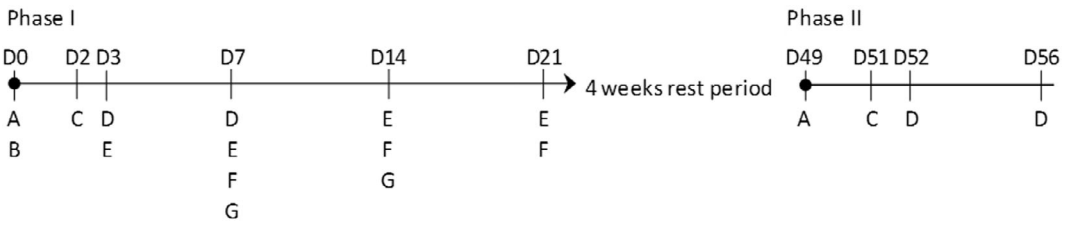

FIGURE 1 Time course of patch testing and repeated open application testing (ROAT) (D = day).

TABLE 1 Subjects recruited by each centre.

Centre	Total recruited	Eugenol positive	Eugenol negative	Patch tested +/++/+++ in phase I	Patch tested +/++/+++ in phase II	ROAT positive
Heidelberg	6	3	3	2	1	1
Bari	5	3	2	0	0	2
Malmö	16	7	9	4	4	3
Odense	20	10	10	4	5	5
Alicante	15	8	7	8	7	6
Barcelona	5	3	2	3	3	2
Total	67	34	33	21	20	19

Abbreviation: ROAT, repeated open application test.

Kemetyl AB, Haninge, Sweden. Eugenol was used at the Department of Occupational and Environmental Dermatology in Malmö, Sweden to make serial dilutions in DEP:ethanol 2:98 w/v for patch testing. The concentration of eugenol in each of the solutions was verified by high-performance liquid chromatography. The DEP:ethanol solutions contained eugenol in the following concentrations in w/v: 2.0%, 1.32%, 1.0%, 0.5%, 0.25%, 0.125%, 0.063%, 0.031%, 0.016%, 0.008%, 0.004%, 0.002%, 0.001%, 0.0005%, 0.00025%, 0.00012% and 0.00006%. This results in an application dose of 600, 396, 300, 150, 75, 37.5, 18.9, 9.3, 4.8, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.036 and 0.018 μg/cm^2, respectively.

The following eugenol dilutions in w/v were used for the ROAT: (a) 2.7%, (b) 1.0%, (c) 0.5% and (d) 0.0% (control). The dose of one application of the ROAT solutions was 114.0 μg/cm^2, 42.2 μg/cm^2, and 21.1 μg/cm^2, respectively. The concentrations for the ROAT solutions were based upon the International Fragrance Association (IFRA) Standard[1] (43rd Amendment, 2008) of 0.5%, which is the highest concentration suggested in any of the categories including skin contact. The 42nd Amendment (2007), listed the highest suggested concentration of 2.7% based on a quantitative risk assessment. The 1.0% dose was selected since it is an intermediate concentration.

2.4 | Patch testing

The patches consisted of 8 mm Finn Chambers® (SmartPractice, Phoenix, Arizona, USA) with 1 filter paper mounted on Scanpor® (Norgesplaster A/S, Oslo, Norway) tape (area of 0.5 cm^2). All test solutions were coded. A volume of 15 μL of test solution was applied to the chambers by a micropipette.[8] The patches were prepared at the individual clinics immediately before application and were not allowed to volatilise prior to application.[9,10] Each subject, whether eugenol-sensitive or negative, was tested with 17 dilutions of 2:98 DEP:ethanol solutions of eugenol as described earlier. The series also included 2:98 DEP:ethanol, neat ethanol and neat DEP as controls. Each subject was patch tested on the upper left and right of the back. The patches were applied for 2 days, visual readings were carried out twice by Dermatologist A on Days 3/4 and 6/7/8, according to the International Contact Dermatitis Research Group classification of patch test reactions.[11] Dermatologist A conducted readings without the knowledge of the test concentration applied, therefore the localisation of each dilution in the patch was randomised for each subject.

2.5 | Repeated open application testing

The ROAT solutions were supplied to participating centres in 8 mL Chemotechnique polypropylene droplet bottles, each containing 3.0 mL of solution. Two test materials were applied to each arm. The test solution droplet bottles were randomised and coded with the letters A, B, C and D to aid the subject during application and so the study was performed in a double-blind fashion. Areas of 3 × 3 cm on the lower volar aspect of the right and left arm were marked with letters corresponding to the coded droplet bottles and used as test sites. Each subject applied the solutions twice a day to the right and left arm for a period of 21 days. Two drops of solution with a weight of about 38 mg were placed on the marked test site and distributed evenly over the test area by use of the tip of the bottle. The tests substances were allowed to dry for 15 min before the test site was allowed to be covered with clothing. The droplet bottles were returned by the subject and weighted every week for evaluation of the test sites and the amount used did not differ substantially between subjects. Each week the subjects were supplied with fresh bottles.

Dermatologist B evaluated the ROAT sites on Days 3, 7, 14 and 21. To ensure each subject's safety, visits with a dermatologist were available at any time during the study at the subject's request. A positive reaction to the ROAT was characterised by clearly visible and infiltrated erythema (with or without papules/vesicles) being present in a minimum of 25% of the application site. Subjects showing unclear reactions to the ROAT continued applications until at least clearly visible and infiltrated erythema was present. In case of a visible positive skin reaction on a test site, the application was stopped on the particular site after reading by Dermatologist B. If the reaction consisted of only itching or infiltrated erythema covering less than 25%, then the subject continued. The application to the other sites was continued until a positive reaction appeared there or until the end of Phase I on Day 21. The control subjects were tested to rule out non-specific reactivity.

2.6 | Ethics committees

Approval was obtained from the ethics committees in the participating countries. A written informed consent was obtained from all subjects. The study was performed in accordance with the Declaration of Helsinki.

2.7 | Statistical calculations

This study was performed at six European dermatology clinics, data analysis was performed in Heidelberg. Statistical analyses were performed using SPSS 25 (©IBM). Fisher's exact test was used for comparison between two groups. The correlations between the number of days until a positive ROAT, threshold patch test concentrations and eugenol concentration of the three ROAT dilutions, respectively, were expressed using the Spearman rank correlation coefficient. Intra-individual comparisons of the test reactions between eugenol solutions and the vehicle were performed using the McNemar test. The chosen level of significance was $p < 0.05$.

The overall threshold concentrations in this sample are reported as the minimum elicitation threshold (MET) for 5% of the test population and the MET for 10% of the test population, based upon the lowest eugenol concentration eliciting a positive patch test. The threshold concentration was defined as the weakest concentration score (reaction) of at least 1+ giving a visible reaction (erythema and infiltration only) on Day 3 or 7 in a nearly continuous line of patch test reactions starting from the highest test concentration. If the positive patch test reactions were not continuous, then if the number of negative and/or doubtful reactions were followed by the same number or more of positive reactions, the lowest positive concentration was determined to be the threshold concentration.[12] In other situations, the concentration eliciting a positive reaction above the highest negative or doubtful reaction was considered to be the threshold concentration.

In order to classify the degree of allergic reactivity to eugenol, the study subjects were grouped into four categories according to their reactivity shown in patch testing: (a) control subjects (no known sensitisation to eugenol), (b) positive patch test to eugenol in the past but not in the present study (in phase I), (c) positive reaction to eugenol in a dilution ≥1.0% in the present study (in phase I) and (d) positive reaction to eugenol in a dilution <1.0% in the present study (in phase I).

3 | RESULTS

3.1 | Patch testing

None of the control subjects tested positively to any of the eugenol dilutions, neither in phase I, nor in phase II. In patch test phase I, 21 (61.8%) out of the 34 test subjects showed a positive reaction (+/++/+++) to at least one of the eugenol dilutions tested (Table 1). The lowest concentration eliciting a positive patch test reaction in phase I was 0.031% (9.3 μg/cm^2) in one subject. In patch test phase II (1 month after the ROAT) a positive reaction to eugenol was found in 20 (58.8%) test subjects. The lowest concentration eliciting a positive patch test reaction in phase II was 0.016% (4.8 μg/cm^2) in one subject.

TABLE 2 Lowest positive patch test reaction (elicitation threshold) in phase I & II.

Positive subjects: Threshold concentration in % (μg/cm^2) of eugenol	Phase I $n = 21$ *n* (%)	Phase II $n = 20$ *n* (%)
0.016% (4.8 μg/cm^2)	0 (0.0%)	1 (5.0%)
0.031% (9.3 μg/cm^2)	1 (4.8%)	0 (0.0%)
0.063% (18.9 μg/cm^2)	0 (0.0%)	2 (10.0%)
0.125% (37.5 μg/cm^2)	4 (19.0%)	2 (10.0%)
0.250% (75 μg/cm^2)	3 (14.3%)	4 (20.0%)
0.500% (150 μg/cm^2)	2 (9.5%)	1 (5.0%)
1.000% (300 μg/cm^2)	7 (33.3%)	3 (15.0%)
1.320% (396 μg/cm^2)	1 (4.8%)	3 (15.0%)
2.000% (600 μg/cm^2)	3 (14.3%)	4 (20.0%)
Mean threshold (SD)	0.79 (0.64)	0.84 (0.75)
Median threshold	0.5	0.5

Three (8.8%) of the 34 test subjects with positive reactions (to a concentration of 2.0%, 1.32% and 1.0, respectively) in phase I tested negatively in phase II while two (5.8%) of the test subjects with a negative reaction in phase I tested positively in phase II (both with a positive reaction to a concentration of 2.0%). The same test reactivity in phase I and II, defined as a negative test reaction or the lowest concentration eliciting a positive reaction, was noted in 16 test subjects while 6 and 7 subjects reacted to a lower concentration in phase I and II, respectively. The mean concentration needed for a positive reaction in phase I was 0.79% and in phase II 0.84% (difference not significant) and the strength of reaction was the same on both occasions.

The MET in patch testing in phase I was 9.3 μg/cm^2 (5%-MET) and 37.5 μg/cm^2 (10%-MET). These thresholds were slightly lower in phase II, in which is 4.8 μg/cm^2 (5%-MET) and 18.9 μg/cm^2 (10%-MET) were observed. Because of the small number of positive patch test results, the 5%-MET is defined by one subject and the 10%-MET by two subjects showing a positive reaction (Table 2).

3.2 | Repeated open application test

None of the 33 control subjects developed a positive ROAT. In the 34 eugenol sensitive test subjects, positive ROAT reactions were noted in 19 (55.9%) to eugenol at 2.7%, in 18 (53.9%) to eugenol at 1.0% and in 7 (20.6%) to the 0.5% solution, respectively. Four test subjects (11.8%) with a negative patch test to eugenol in phase I developed a positive ROAT to eugenol at 2.7% and three of these four subjects reacted positively also to the ROAT solution with

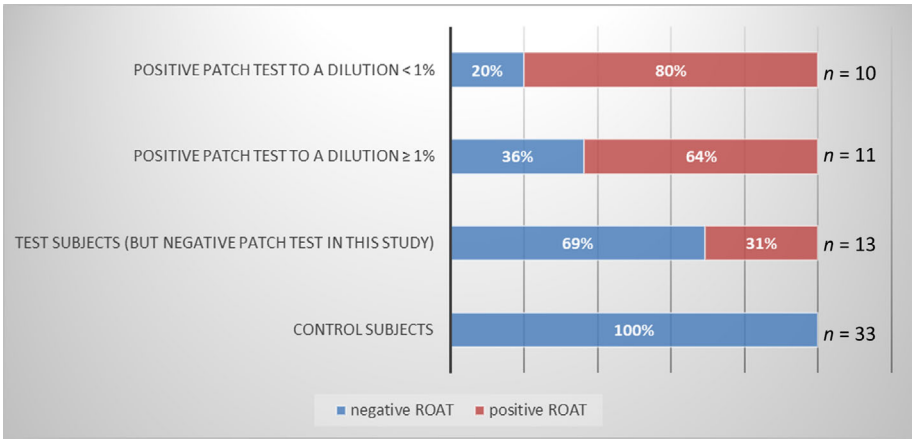

FIGURE 2 At least one positive repeated open application test (ROAT) with eugenol by patch test reactivity in phase I.

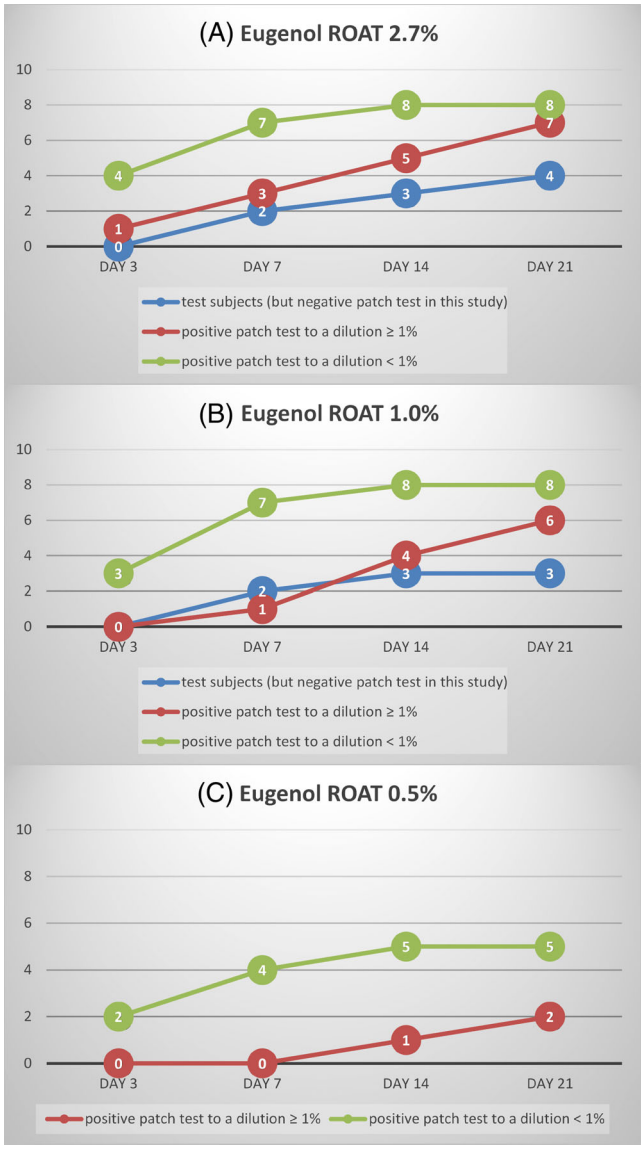

FIGURE 3 Time until a positive repeated open application test (ROAT) with dilutions of eugenol at (A) 2.7%, (B) 1.0% and (C) 0.5% by patch test reactivity in phase I.

eugenol at 1.0%. None of the four tested positively to the ROAT solution with eugenol at 0.5%. One test subject (2.9%) developed a positive ROAT to the vehicle. The comparison between control and test subjects concerning the number of positive ROATs for the three ROAT solutions with eugenol at 2.7%, 1.0% and 0.5% resulted in *p*-values at <0.0001, <0.0001 and <0.0111, respectively. In the test subjects, the intra-individual comparison performed for the respective ROAT solution with eugenol at 2.7%, 1.0% and 0.5% regarding the number of positive ROATs registered for the ROAT solution compared to the vehicle resulted in *p*-values at <0.0001, <0.0001 and 0.0233, respectively.

Figure 2 shows the association between degree of patch test reactivity to the dilutions of eugenol in phase I and a positive ROAT. The lower the patch test concentration of eugenol eliciting a positive test reaction, the more likely a positive ROAT (Spearman's roh = 0.7; $p < 0.001$). All but one of test subjects (90%) with an elicitation threshold lower than 1.0% ($n = 10$) developed a positive ROAT to the ROAT solutions with eugenol at 2.7% and 1.0% while the corresponding figure for the ROAT solution with eugenol at 0.5% was 5 of these 10 subjects (50%). Figure 3A–C demonstrates the association between degree of patch test reactivity to the dilutions of eugenol and first day of appearance of a positive ROAT. The lower the patch test concentration of eugenol eliciting a positive test reaction, the more likely a positive ROAT appears early (Spearman's roh = 0.5; $p < 0.05$). Only one (9.1%) of the 11 test subjects with an elicitation threshold at 1.0% or higher at patch testing had developed a positive ROAT to the ROAT solution with eugenol at 2.7% on Day 3 while 4 (40%) of the 10 subjects with an elicitation threshold lower than 1.0% had developed a positive ROAT on Day 3 (Figure 3A).

4 | DISCUSSION

Many usage tests have been performed with fragrance sensitisers. The proportion of positive reactions varies between 0% and 100%.[13–26] Concentration, actually dose/cm^2, of the applied usage/ROAT preparation and length of application period are major reasons

for the great variation. In a patch test and ROAT study on eugenol, none of 30 eugenol-hypersensitive subjects developed a positive reaction to a ROAT solution with eugenol at 0.5%.[21] A similar pilot study was therefore performed in five eugenol-hypersensitive subjects with the same eugenol concentrations in the ROAT solutions as for the present study.[22] Again, none of these five subjects developed a positive ROAT to the solution with eugenol at 0.5% while four (80.0%) and one subject (20.0%) developed a positive ROAT to the solutions with eugenol at 2.7% and 1.0%, respectively.[22] Notably from the pilot study[22] is that one subject (20%) with a previous positive patch test to eugenol at retesting had negative patch test reactions to all concentrations tested but still developed a positive ROAT, but only to the ROAT solution with eugenol at 2.7%.[22] In the present study, 4 out of 34 test subjects (11.8%) tested negatively to eugenol at patch testing in phase I but still developed positive ROATs to the solutions at 2.7% and 3 of those (8.8%) also to 1.0% but none to 0.5%. However, the most recent IFRA standard (50th amendment 2022) listed the highest suggested concentration of 2.5% in products with skin contact. Therefore, the ROAT results with the 2.7% eugenol may be relevant for clinical practise: patients with a positive patch test reaction should avoid parfums containing eugenol for daily use. However, these results cannot be generalised to other (fragrance) allergens, here separate studies are needed.

Positive ROATs were obtained in 55.9%, 52.9% and 20.6% of the 34 test subjects to the ROAT solutions with eugenol at 2.7%, 1.0% and 0.5%, respectively, while none of the control subjects developed any positive ROAT. The difference in the number of positive ROATs between test and control subjects is statistically significant for all three eugenol concentrations in the ROAT solutions, which rules out irritancy as the cause of the positive ROAT. Furthermore, the fact that only one test and no control subject developed a positive ROAT to the ROAT vehicle when applied for 3 weeks in all subjects demonstrates that the positive ROATs are manifestations of allergic contact dermatitis from eugenol. In the test subject with a positive ROAT to the vehicle, the vehicle droplet bottle might have been accidentally interchanged by this subject. The fact that 44.1% of the 34 test subjects hypersensitive to eugenol did not develop a positive ROAT indicates that they can use scented products containing eugenol on non-damaged skin without getting skin problems at least for 21 days, particularly if the products are used less frequently than in this study. However, the situation may be different if products such as scented moisturisers are applied on skin with an existing dermatitis[5] or damaged skin barrier or under occlusion.

There were 14 out of the 34 test subjects (41%) with a previous positive patch test reaction to eugenol who had a negative reaction in phase I in the present study. This might in part be caused by testing with DEP:ethanol as vehicle, while the previous patch test was performed in petrolatum. Three of the test subjects with positive patch test reactions in phase I to eugenol at 2.0%, 1.32% and 1.0%, respectively, tested negatively in phase II and two negative test subjects in phase I tested positively to eugenol at 2.0% in phase II. Previous false positive reactions to eugenol may be one explanation. However, another explanation is a variation in test reactivity over time. In approximately half the number of test subjects, the elicitation threshold varied between phase I and phase II with either a higher (7 subjects) or lower (6 subjects) threshold to eugenol. A variation in test reactivity over time has previously been reported,[12,27–30] as well as a higher risk for weak allergic reaction to vary between positive and negative patch test reactions when multiple patches with the same sensitiser are applied on the same occasion.[31]

Expectedly, there was an association between degrees of hypersensitivity at patch testing and the outcome of the ROAT. The stronger reaction at patch testing in phase I, defined as the lowest eugenol dilution eliciting a positive patch test, the more likely was a positive ROAT, and the more likely it appeared early during the application period (Figure 3A–C). Eight subjects (80%) of those reacting positively at patch testing to lower eugenol concentrations than 1.0% ($n = 10$) developed a positive ROAT (Figure 2). For these test subjects, 50% of the positive ROATs to the ROAT solution with eugenol at 2.7% had appeared by the D3 reading (Figure 3). This kind of relationship has previously been demonstrated for other fragrance sensitisers including isoeugenol,[6,17] hydroxyisohexyl 3-cyclohexene carboxaldehyde[11,18] and oak moss[23,24] and was also reported in the ROAT study with FM I and FM II.[2,26]

According to a safety assessement the non expected sensitisation induction level (NESIL) of eugenol is a dose of 5900 μg/cm^2.[21] Even by considering the small sample size in this study, it was shown that the METs in patch testing (2 days under occlusion) is lower than the NESIL. However, it must be realised that the NESIL is geared to prevent induction of new sensitisation, and is not a level necessarily considered safe for elicitation in those already sensitised, as is shown here.

In conclusion, the patch test reactivity to eugenol was on an average, virtually the same on the two test occasions 2 months apart. False-negative reactions were noted on both occasions. In this experimental set-up with a ROAT during 3 weeks, significant numbers of positive ROATs were obtained with the three ROAT solutions with eugenol at 2.7%, 1.0% and 0.5%. It was demonstrated that 80% of those with contact allergy to eugenol did not develop a positive ROAT to the ROAT solution with eugenol at 0.5%. The stronger the patch test reactivity, defined as the lowest eugenol dilution eliciting a positive patch test reaction, the more likely was a positive ROAT and the more likely it was that the positive ROAT appeared early during the application period. Subjects with a previous positive patch test reaction followed by a negative reaction to eugenol at the start of the ROAT may still develop a positive ROAT.

AUTHOR CONTRIBUTIONS

Robert F. Ofenloch: Methodology; software; data curation; formal analysis; visualization; writing – original draft; project administration. **Klaus Ejner Andersen:** Methodology; data curation; supervision; writing – review and editing; resources. **Caterina Foti:** Data curation; supervision; resources; writing – review and editing; investigation. **Ana Maria Giménez-Arnau:** Data curation; supervision; writing – review and editing; investigation; resources. **Martin Mowitz:** Data curation; methodology; investigation; writing – review and editing.

Juan Francisco Silvestre Salvador: Data curation; supervision; resources; investigation; writing – review and editing. **Cecilia Svedman:** Data curation; supervision; resources; writing – review and editing; investigation. **Magnus Bruze:** Conceptualization; data curation; supervision; resources; methodology; formal analysis; writing – original draft; project administration; funding acquisition; validation.

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CONFLICT OF INTEREST STATEMENT

Magnus Bruze is a member of the Expert Panel for Fragrance Safety—http://fragrancesafetypanel.org/. The other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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ENDNOTE

[1] All IFRA standards accessed at https://ifrafragrance.org/safe-use/standards-documentation, 15 December 2022.

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