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INVESTIGATIVE REPORT

Usage Tests of Oak Moss Absolutes Containing High and Low Levels of Atranol and Chloroatranol

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Atranol and chloroatranol are strong contact allergens in oak moss absolute, a lichen extract used in perfumery. Fifteen subjects with contact allergy to oak moss absolute underwent a repeated open application test (ROAT) using solutions of an untreated oak moss absolute (sample A) and an oak moss absolute with reduced content of atranol and chloroatranol (sample B). All subjects were in addition patch-tested with serial dilutions of samples A and B. Statistically significantly more subjects reacted to sample A than to sample B in the patch tests. No corresponding difference was observed in the ROAT, though there was a significant difference in the time required to elicit a positive reaction. Still, the ROAT indicates that the use of a cosmetic product containing oak moss absolute with reduced levels of atranol and chloroatranol is capable of eliciting an allergic reaction in previously sensitised individuals. Key words: oak moss absolute, atranol, chloroatranol, repeated open application test (ROAT), contact allergy.

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Oak moss absolute (OMA) with the International Nomenclature of Cosmetic Ingredients (INCI) name *Evernia prunastri* extract is a fragrance ingredient derived from the lichen *E. prunastri*. OMA is included in Fragrance mix I (FM I) used in routine patch testing to diagnose fragrance contact allergy and has been reported as the most common allergen among the fragrances in FM I (1).

Atranol and chloroatranol, two degradation products formed during the manufacturing of OMA, have been identified as main allergens in OMA (2). The content of atranol and chloroatranol in untreated OMA has been reported to be in the range of 2.4–2.9 % and 0.9–1.4%, respectively (2–4).

According to the Cosmetic Products Regulation of the European Union, OMA has to be declared with its INCI name when present in leave-on products at levels above 10 ppm and when present in rinse-off products at levels above 100 ppm. The Cosmetic Products Regulation does not regulate the levels of oak moss extracts

in cosmetic products or the levels of chloroatranol and atranol in the extracts (5). According to the International Fragrance Association (IFRA) Standard on oak moss extracts, the maximum concentration allowed in skin contact cosmetic products is 0.1%. Since 2008 there is also an IFRA restriction on the concentration of atranol and chloroatranol in oak moss extracts, which must not exceed 100 ppm each (6).

In 2004, the Scientific Committee on Consumer Products (SCCP), an independent advisory committee to the European Commission, recommended that atranol and chloroatranol should not be present in cosmetic products (7). When reviewing sensitisation data on treated and untreated OMA samples in 2008, the SCCP concluded that it appears to be possible to reduce the content of atranol and chloroatranol to <2 ppm each. A cosmetic product containing 0.1% OMA would then contain atranol and chloroatranol in such levels that the risk of both induction and elicitation of allergic reactions would be low. However, the SCCP expressed the need of appropriate clinical testing with treated OMA samples in subjects previously sensitised to OMA in order to demonstrate a reduction in the elicitation capacity (8).

OMA with a reduced content of atranol and chloroatranol has been demonstrated to be able to elicit positive patch test reactions in individuals previously diagnosed with contact allergy to OMA (9, 10), but the ability of treated OMA to elicit allergic reactions upon repeated skin exposure is not known. Therefore, we investigated the eliciting properties of a treated and an untreated OMA in a repeated open application test (ROAT) and in patch tests with serial dilutions.

MATERIAL AND METHODS

Study population

Fifteen subjects (13 women, 2 men, mean age 54 years, range 34–68 years) diagnosed with contact allergy to OMA at our department during 2007–2011 were enrolled in the study. The strength of the original patch test reactions to OMA was scored as + in 5 subjects, as ++ in 5 subjects and as +++ in 5 subjects. In addition, 16 controls (13 women, 3 men, mean age 55 years, range 31–69 years) without contact allergy to fragrances or *Myroxylon pereirae* were included in the study. Exclusion criteria for the study were ongoing dermatitis on any of the test sites and treatment with systemic corticosteroids. During the study the participants were asked not to use any topical corticosteroids or personal care products on the test sites on the arms.

Chemicals and test preparations

Patch test and ROAT solutions were prepared at our department in a vehicle similar to those used in fine fragrances. The vehicle consisted of 2.0% (v/v) diethyl phthalate (DEP) (Sigma Aldrich, Steinheim, Germany) and 98.0% (v/v) ethanol (95%, Kemetyl AB, Haninge, Sweden). A sample of a traditional untreated OMA (sample A) was provided by manufacturer I. Solutions of sample B containing equal amounts of 3 IFRA compliant OMA samples with reduced levels of atranol and chloroatranol (from manufacturer I, II and III) were prepared from solutions of the individual samples.

Samples A and B were patch-tested in dilution series with the same dilution steps as in a previous ROAT study on eugenol (11). Stock solutions (2.0% w/v) of samples A and B were further diluted by a factor of 2 to the following concentrations: 1.0, 0.50, 0.25, 0.13, 0.063, 0.031, 0.016, 0.0078, 0.0039, 0.0020, 0.00098, 0.00049, 0.00024, 0.00012 and 0.000061% (w/v). To improve the sensitivity an extra dilution step at 1.3% (w/v) was included between 2.0% and 1.0%. In addition, pure DEP and ethanol as well as the mixture of DEP and ethanol (2:98) were included in the patch test series. ROAT solutions of samples A and B were each prepared in a concentration of 0.10% (w/v). Furthermore a sample of the vehicle and a 0.00020% (w/v) dilution of sample A with atranol and chloroatranol concentrations in the same order of magnitude as in the 0.10% preparation of sample B were used in the ROAT.

Study design

All participants were patch-tested in connection to the start-up of the ROAT. The study was conducted in a double-blinded fashion and the patch tests and ROATs were performed and read as described previously (11). The ROAT was performed on 4 3×3 cm sites, 2 on the lower volar aspects of each arm. The corners of the squares were marked with a surgical marker pen. The squares as well as the 8 ml polypropylene droplet bottles (Chemotechnique, Vellinge, Sweden) containing the ROAT solutions were coded as A, B, C and D. The participants were instructed on how to apply the solutions and to allow the solutions to dry before putting on clothing. Two droplets (about 40 mg) of each solution were applied twice daily and the solutions were distributed evenly on the marked sites with the tip of the bottle. The ROATs were read after 3, 7, 14, 21, and 28 days. The ROAT was regarded as positive when at least 25% of the test area was covered with erythema, infiltration and papules. When a reaction was graded as positive, the participant was instructed to stop application to the site where the reaction had occurred and to continue with the application of the solutions to the other sites. Every week the used bottles were exchanged to fresh ones containing the same solutions. The bottles were weighed before and after usage in order to achieve an estimate of the amount applied to the test sites.

Ethics

The study was approved by the Regional Ethical Review Board in Lund, Sweden. Written informed consent was obtained from each participant.

Statistics

Fisher's exact test (two-sided) was used when comparing the number of subjects and controls reacting positively to the patch

tests and the ROATs. McNemar's test (two-sided) was used to compare the number of oak moss-allergic subjects reacting to samples A and B in the patch tests and ROATs. McNemar's test was also used to compare the reactivity, expressed as the minimum eliciting concentration (MEC), to the patch tests of samples A and B and also for comparison of the time required for elicitation of a positive ROAT reaction. Differences were considered significant at $p < 0.05$.

The positive patch test reactions were not always continuous. When the number of negative and/or doubtful reactions was followed by at least the same number of positive reactions, the lowest positive reaction was considered the MEC (12). If negative or doubtful reactions at 2.0% and 1.3% were followed by a positive reaction at 1.0% (as in subject 3), the latter was registered as the MEC. Otherwise the last positive concentration above the negative or doubtful reactions was considered the MEC. In the calculations of the ratio of the MEC of sample A and sample B it was assumed that subjects negative to sample A and/or sample B would test positively to these samples in a concentration of 4.0% (w/v), i.e. the highest concentration in the dilution series multiplied by a factor of 2. The correlation between the reactivity in the patch test of sample A and the reactivity in the ROAT of samples A and B was assessed using Spearman rank correlation. In these calculations the patch test reactivity, expressed as the MEC, and the ROAT reactivity, expressed as the number of days until observation of a positive reaction, was ranked. The higher the reactivity, the lower the rank number. Subjects with negative patch tests and/or ROATs, i.e. those showing the lowest reactivity, were given the highest rank number.

RESULTS

Patch tests

The outcome of the patch tests is summarised in Table I and Table SI¹. There was a statistically significant difference in the number of subjects reacting to samples A and B ($p = 0.031$). Thirteen subjects were found to be more reactive towards sample A, and 2 were equally reactive to samples A and B ($p < 0.001$).

The MEC of sample A ranged between 0.00049% and 2.0% and the MEC of sample B ranged between 0.13% and 2.0%. The ratio between the MEC of sample B and sample A varied between 1 and 2,000 in the individual

Table I. Number of oak moss-allergic subjects and controls reacting to samples A and B in patch tests and in a repeated open application test (ROAT)

Sample	Positive/Tested, <i>n</i>		
	Oak moss-pos group	Control group	Oak moss-pos vs. controls ^a
Patch tests			
A	14/15	0/16	$p < 0.001$
B	8/15	0/16	$p < 0.001$
ROAT			
A 0.10 %	11/15	0/16	$p < 0.001$
A 0.00020%	8/15	0/16	$p < 0.001$
B 0.10%	8/15	0/16	$p < 0.001$
Diethyl phthalate/ethanol 2:98	0/15	0/16	$p > 0.3$

^aFisher's exact test (two-sided).

¹<http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1725>

subjects. One subject showed a + reaction to ethanol, but was negative to 2:98 DEP/ethanol. No reactions were observed to the pure DEP or 2:98 DEP/ethanol. Three reactions to sample A and 2 reactions to sample B, which were doubtful on D3 were scored as positive on D7.

ROAT

The time required for elicitation of allergic reactions is given in Table I and Table SI¹. Fig. 1 shows a positive ROAT to sample B in subject 12. There were no statistically significant differences between the number of subjects reacting to samples A and B at 0.10% ($p=0.25$), sample A at 0.10% and 0.00020% ($p=0.25$) or sample A at 0.00020% and sample B at 0.10% ($p>0.3$). However, a significant difference was observed when comparing the number of days until observation of a positive reaction after exposure to sample A at 0.10% and sample B at 0.10%. Ten subjects were found to react earlier to sample A than sample B and 5 were equally reactive to samples A and B ($p=0.0020$). Similarly, there was a significant difference when comparing the reactivity to the 0.10% and 0.00020% preparations of sample A. Eight subjects were more reactive towards the 0.10% preparation and 7 were equally reactive ($p=0.0078$). No statistically significant difference was found between the reactivity to sample A at 0.00020% and sample B at 0.10% ($p>0.3$).

Fig. S1¹ illustrates the relationship between the patch test reactivity towards sample A and the outcome of the ROATs. Correlations were found between the MEC of sample A and the number of days until a positive reaction to the ROAT of sample A at 0.10% ($r=0.85$, $p<0.001$), the MEC of sample A and the number of days until a positive reaction to the ROAT of sample A at 0.00020% ($r=0.76$, $p=0.0011$), and between the MEC of sample A and the number of days until a positive reaction to the ROAT of sample B at 0.10% ($r=0.86$, $p<0.001$).

The oak moss-allergic subjects applied a mean of 140 mg/day (range 88–230) of the ROAT solutions to each test site and the controls applied a mean of 130 mg/day (range 86–230).



Fig. 1. A positive reaction to the repeated open application test of sample B (treated oak moss absolute) at 0.10% (w/v) in subject 12 on day 14.

DISCUSSION

The raw material used for OMA is the lichen *E. prunastri*, which is collected from oak trees in the south-central regions of Europe as well as in Morocco and Algeria. Each year about 700 tons of the lichen is processed in France. After the harvest the lichen is desiccated and then humidified with water prior to the extraction procedure with organic solvents. The solvents used are either hexane or mixtures of hexane and more polar solvents, mainly acetates. The crude solvent extracts, called resinoids, are further treated with ethanol in order to obtain the absolutes, which are then used in fragrance compositions. The absolutes may in addition be subjected to physical treatments such as discolouration with charcoal or high-vacuum distillation (4). The chemical composition of natural extracts is often complex and more than 170 substances have been identified in oak moss extracts. Some of these substances are formed during the processing of the extracts. When the dried lichen is treated with water, phenyl benzoate derivatives such as atranorin and chloroatranorin are hydrolysed and further decarboxylation results in the formation of atranol and chloroatranol (4, 13).

Several substances found in lichens have been reported as contact allergens including atranorin, evernic acid, fumarprotocetraric acid and usnic acid (14–15). More recently, atranol and chloroatranol have been identified as strong allergens and methyl β -orcinolcarboxylate has been identified as a weak allergen (2).

Chloroatranol has been demonstrated to cause allergic reactions at ppb level in patch tests and at ppm level in a ROAT (17). The elicitation capacity of chloroatranol has been identified as 2.2 times higher than that of atranol. However, the concentration of atranol in OMA is about twice as high as the concentration of chloroatranol (18). In 2004, Rastogi et al. (19) found atranol and chloroatranol in 27/31 investigated products, mainly perfumes. The median concentration in perfumes was 0.50 ppm for atranol and 0.24 ppm for chloroatranol. They concluded that these sources of exposure could explain the high frequencies of OMA contact allergy. In 2007, a significant decrease in the proportion of products containing chloroatranol was observed compared to the aforementioned study (20).

The content of atranol and chloroatranol in the IFRA compliant OMA offered on the market today is below 100 ppm each. Consumers using cosmetic products containing these extracts are exposed to atranol and chloroatranol in concentrations of 0.1 ppm or below. There are several methods reported on how to reduce the content of sensitizers in OMA involving e.g. chromatographic methods (21), treatment with amino acids (3) or binding to an insoluble polymer support (9), though it is not likely that any of these methods specifically removes atranol and chloroatranol.

Nardelli et al. (9) performed patch testing with a 1% petrolatum preparation of OMA treated with a polymer-based method which reduced the content of atranol and chloroatranol to <75 ppm and <25 ppm, respectively. Still, 8 out of 14 oak moss-allergic individuals reacted to the treated sample and the authors concluded that the described treatment “reduces the allergenic elicitation potential in previously sensitised individuals only to a minor extent” and that the residual amounts of atranol and chloroatranol are “unsafe for the consumer”.

We observed a similar result in our patch tests where 14/15 reacted to the untreated quality (sample A) and 8/15 reacted to the treated quality (sample B). In another study, where patch testing with serial dilutions of a treated and an untreated sample of OMA was performed, we observed reactions to the treated sample at 2.0% in acetone in only 2/15 oak moss-allergic subjects, while all 15 reacted to the untreated sample at 2.0% or below (10). However, in the aforementioned study samples from different producers and batches were used. The applied daily dose of the ROAT solutions to each test site was approximately the same for the oak moss-allergic group (140 mg) and the control group (130 mg). However, both groups consumed more of the solutions than the intended 80 mg/day. Positive ROATs were observed in 11 subjects with the untreated quality (sample A) at 0.10% and in 8 subjects with the treated quality (sample B) at 0.10%. All subjects reacting to sample B also reacted to sample A at 0.10%. There was a statistically significant difference in the time of exposure required to elicit allergic contact dermatitis between samples A and B when tested at 0.10%. Three subjects (Nos. 1, 5 and 13) were positive in the ROAT of sample B but were not considered positive to the patch tests of sample B. Subject 1 had a doubtful reaction at 2.0% and + reactions at 0.25% and 0.031%, but was not considered positive since there were several negative reactions above the 1st positive reaction in the dilution series. In subject No. 5 the patch test of sample B at 2.0% were interpreted as irritant, while no patch test reactions of any kind were observed for sample B in subject 13. In subjects 1, 5 and 13 the content of the bottles had been randomised in such a way that sample B and the vehicle were applied to one arm and the 2 dilutions of sample A were applied to the other. Thus, the risk of false-positive reactions to the ROAT of sample B due to spill of sample A or spreading of eczematous reactions to sample A onto the area where sample B was applied could be ruled out.

The positive reactions to the patch test and ROAT of sample B could be explained either by reactions to the low levels of atranol and chloroatranol and/or by reactions to other substances present in the sample. The ROAT was performed with 2 concentrations of sample A, 0.10% and a 500-fold dilution of the 0.10% preparation, i.e. 0.00020%, which reflects the ratio by which the content of atranol and chloroatranol is reduced in a treated OMA. Among the 9 subjects with a positive

ROAT to either sample A at 0.00020% or sample B at 0.10%, one reacted exclusively to sample A and one reacted exclusively to sample B. The similarity in reaction pattern indicates that the residual levels of atranol and chloroatranol are responsible for the allergic reactions. The levels of these substances are in the same order of magnitude both in sample A at 0.00020% and in sample B at 0.10%, while the levels of substances which are not affected by the treatment of the absolutes would be considerably higher in the 0.10% preparation of sample B.

To conclude, we have observed a significant difference in the reactivity towards the treated and untreated samples of OMA. It is therefore likely that the treated sample is also less prone to induce sensitisation. Still 8/15 oak moss-allergic subjects showed an allergic reaction in the ROAT of sample B at 0.10%. This indicates that a cosmetic product containing 0.10% of an OMA with a similar composition as sample B is capable of eliciting contact allergic reactions in individuals previously sensitised to OMA.

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Conflicts of interest: MB is a member of the expert panel of RIFM, an independent group of experts who evaluate the safety of fragrance materials, which is supported by the manufacturers of fragrances and consumer products containing fragrances. MM, CS and EZ declare no conflict of interest.

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