Reduced content of chloroatranol and atranol in oak moss absolute significantly reduces the elicitation potential of this fragrance material

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Summary

Background. Oak moss absolute, an extract from the lichen *Evernia prunastri*, is a valued perfume ingredient but contains extreme allergens.

Objectives. To compare the elicitation properties of two preparations of oak moss absolute: 'classic oak moss', the historically used preparation, and 'new oak moss', with reduced contents of the major allergens atranol and chloroatranol.

Patients/materials/methods. The two preparations were compared in randomized double-blinded repeated open application tests and serial dilution patch tests in 30 oak moss-sensitive volunteers and 30 non-allergic control subjects.

Results. In both test models, new oak moss elicited significantly less allergic contact dermatitis in oak moss-sensitive subjects than classic oak moss. The control subjects did not react to either of the preparations.

Conclusions. New oak moss is still a fragrance allergen, but elicits less allergic contact dermatitis in previously oak moss-sensitized individuals, suggesting that new oak moss is less allergenic to non-sensitized individuals.

Key words: allergic contact dermatitis; fragrances; patch test; risk assessment.

Oak moss absolute (INCI name *Evernia prunastri*), a natural extract of the aerial parts of oak moss, used to deliver the scent of 'walking in an old forest' in cosmetic products, is a valuable perfume ingredient but also contains extreme contact allergens. Oak moss is part of fragrance mix I used in the European baseline patch test series.

As a natural extract, oak moss absolute is a complex mixture of chemicals, including several allergens (1). The main allergens are atranol and chloroatranol, two degradation products formed during manufacture (1, 2).

After review of available data, the Scientific Committee on Consumer Products (SCCP) concluded in 2004 that atranol and chloroatranol should not be present in cosmetic products (3).

In 2008, preparations of oak moss with lowered atranol and chloratranol contents were assessed by the SCCP. In the resulting SCCP opinion, it is stated that: 'appropriate clinical studies on individuals with characterised

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Conflicts of interest: Magnus Bruze is a member of REXPAN, an expert panel of the Research Institute for Fragrance Materials (RIFM). Armand Bernois, Christophe Brault, Hervé Eudes, Catherine Gadras, Anne-Cécile Jousseau Signoret, Boris P. Müller and Bernard Toulemonde are employees of sponsor companies as listed above. Klaus Ejner Andersen is advisor to SmartPractice, Denmark. Flemming Andersen, Kirsten Hammond Andersen and Kristian Fredløv Mose declare no conflicts of interest.

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contact allergy to Oak Moss are required to demonstrate the dose response characteristics of allergic elicitation reactions with the modified preparations' (4).

In a patch test model, it was shown that that the elicitation potential was reduced with a reduction of the allergen content in oak moss absolute (5, 6). Patch testing, however, does not accurately reflect the daily use of scented products. To remedy this, a pilot study was devised to determine the frequency of elicitation of allergic contact dermatitis by a new preparation of oak moss absolute [with lowered atranol and chloroatranol contents – new oak moss (NOM)], and to compare this with the frequency of elicitation by the 'classic' preparation of oak moss absolute [containing higher levels of atranol and chloroatranol – classic oak moss (COM)]. The study consisted of an initial repeated open application test (ROAT) and a serial dilution patch test at the end of the study.

NOM is formulated in compliance with the purity requirements of the respective International Fragrance Association (IFRA) Standard on Oak Moss extracts published as part of the 43rd Amendment in 2009 (restricting the levels of atranol and chloroatranol to 100 ppm maximum each in the moss extract) at the maximum level allowed by the IFRA Standard (0.1% of oak moss extracts in hydroalcoholics). On the other hand, COM contains atranol and chloroatranol at significantly higher levels. In the pilot study, significantly more subjects reacted in the patch tests with COM than in those with NOM. However, no difference was observed in the ROAT, although there was a significant difference in the time required to elicit a positive reaction. It was concluded that there were significant differences in the elicitation capacities of the treated and non-treated oak moss absolutes, and that it is therefore likely that the treated sample is less prone to induce sensitization. Also, a cosmetic product containing oak moss absolute with reduced levels of atranol and chloroatranol is less capable of eliciting an allergic reaction in previously sensitized individuals (7).

The trial reported here was conducted in order to elucidate in more depth the differences between the two compounds.

Materials and Methods

Volunteers

A total of 60 adults recruited via the Allergen Bank Database (8), living within 1 hr driving distance of Odense, were invited to participate voluntarily: these comprised 30 subjects with a history of previous allergic contact dermatitis and sensitivity to oak moss, and 30 control subjects with a history of previous contact dermatitis but negative patch test results with perfumes, oak moss or tree moss extracts (*Evernia furfuracea*), balsam of Peru (*Myroxylon pereirae*), and/or fragrance mix.

All subjects were informed both verbally and in writing, and had given their informed consent and filled in a medical history questionnaire including questions on alleged skin complaints resulting from the use of perfumed consumer products. The study was approved by the Ethical Committee for the Region of Southern Denmark, S-20120142.

Chemicals

The solutions for the ROAT and patch tests were prepared by Department of Occupational and Environmental Dermatology, Skåne University Hospital, Lund University, Malmö, Sweden.

The solutions for the ROAT were delivered in coded droplet bottles (A, B, C, and D) and kept refrigerated and protected from light. The solutions were delivered in blinded sets of four bottles, one set for each subject, and a new set for each week in order to ensure that the ROAT could be performed double-blinded. The same type of vehicle was used in all tests: 2% diethyl phthalate/98% 95-proof ethanol (2:98 DEP/EtOH).

Each set of bottles contained the following:

1. 0.1% COM containing 27 000 ppm atranol and 15 000 ppm chloroatranol in the neat extract (Certificate of Analysis, Robertet Grasse).
2. 0.1% NOM containing 48 ppm atranol and 37 ppm chloroatranol each in the neat extract (Certificate of Analysis, Robertet Grasse).
3. Vehicle.
4. Vehicle.

For the serial dilution patch tests, the solutions were delivered and kept refrigerated and protected from light in the original packaging. The following 12 concentrations of both COM and NOM were prepared in the vehicle: 2.0%, 1.32%, 0.67%, 0.22%, 0.074%, 0.025%, 0.0082%, 0.0027%, 0.00091%, 0.0003%, 0.0001%, and 0.00003% (wt/vol). Three control solutions with vehicle (2:98 DEP/EtOH), 95-proof ethanol and 100% DEP were also used.

Study design

The protocol was divided in two phases adapted in part from previous studies (7, 9, 10).

In phase I, the ROAT was performed over a 21-day period. The period of 3 weeks was chosen as a practical compromise. We expected, on the basis of previous fragrance studies (9), that a difference between two closely

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related allergens could be detected, and at the same time subjects' adherence to the study protocol would be maintained. After the ROAT period, the subjects had a 4-week rest period, and then they participated in phase II, consisting of the serial dilution patch test. Investigator A read the patch test results, and investigator B read the ROAT results.

Phase I ROAT. Each subject used 0.1% (wt/vol) COM and 0.1% (wt/vol) NOM, and two samples of the vehicle control. The droplet bottles were randomized in a double-blinded fashion. Two areas of $5 \times 5\,cm^2$ on the volar aspects of the right and left arm were marked and used as test sites, so that each subject applied two solutions to each volar forearm.

At the start of the ROAT (day 0), the volunteers were given four weighed and coded (A–D) droplet bottles with the four test materials.

The subjects applied two drops of the solutions twice daily to the marked test site for a period of 21 days or until a positive reaction developed at a test site.

The droplet bottles were collected and weighed each week on days 7, 14, and 21, and replaced with fresh bottles at the weekly clinical evaluation of the test sites. Subjects filled in diaries to confirm the application of product to the areas in question.

In the case of a suspected positive reaction at a test site, the subject attended for an extra evaluation. A positive reaction in the ROAT at a test site was characterized by clearly visible and infiltrated dermatitis (with or without papules/vesicles) being present on a minimum of 25% of the area (10), and the application was stopped on the particular site after reading. The application of test solution to the other three sites continued until a positive reaction appeared there or until the end of phase I on day 21.

Phase II patch test. Each subject, whether previously tested as oak moss-sensitive or as a control, was patch tested with the 12 dilutions of COM and NOM and the three control solutions. The patches were applied 4 weeks after the completion of phase I, and they were randomly applied by use of the randomization engine EDGAR (11).

Eight-millimetre Finn Chambers® (SmartPractice, Phoenix, AZ, USA) containing one filter paper and mounted on Scanpor® tape (Norgesplaster A/S, Oslo, Norway) were used. A volume of 15 µl ($5.9\,mg/cm^2$) of test solution was applied to the chambers with a micropipette immediately before application on the upper left and right sides of the back. The patch sites were marked at the time of application. The patch tests were removed by the investigator after 2 days. Visual readings were carried out by investigator A on day 3 and say 7 after application, according to standard criteria as follows: NT, not tested; −, negative; ?+, doubtful reaction with erythema only; +, weak positive reaction with erythema, infiltration, and possibly papules; ++, strong positive reaction with papules and vesicles; and +++, extreme positive reaction with intense erythema, infiltration, and coalescing vesicles.

Application and reading of the reactions were performed in a double-blinded fashion.

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 day 7 in a graded line of patch test reactions starting from the highest test concentration.

If the positive patch test reactions were not continuous, the lowest positive concentration was considered to be the threshold concentration, provided that at least the same number of positive reactions followed the negative and/or doubtful reactions. If not, the last positive reaction above the negative or doubtful reaction was considered to be the threshold concentration (12).

Statistics

After completion of the investigation, the blinding was broken and the results were evaluated statistically.

The chosen level of significance was $p < 0.05$. Depending on data sets the following statistical methods were used: Wilcoxon test, Friedman test with Dunn's multiple comparisons test or McNemar's test with the continuity correction.

Statistical calculations were performed with GRAPHPAD INSTAT (GraphPad Software, Inc. La Jolla, USA) and the GraphPad McNemar test.

For oak moss-sensitized subjects, if there was no response in the ROAT, 200 applications were arbitrarily chosen as the number necessary to elicit a positive response. If there was no response in the patch tests, a threshold concentration of 20% was arbitrarily chosen. The relationship between patch test threshold doses and responses was analysed with maximum likelihood methods by the use of log-logistic dose–response models (13, 14).

Results

All 60 volunteers completed the test series. Raw data are shown in Tables 1 and 2.

Demographics and medical history

There were 19 female and 11 male subjects in the oak moss-sensitive group, and 24 female and 6 male subjects

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Table 1. Raw data for oak moss-sensitized subjects, sorted according to original patch test response in the database

Participant		4		15		28		32		33		35		36		37		44		3		5		30		11		19		21	
Original patch test		+		+		+		+		+		+		+		+		+		++		++		++		++		++		++	
Reaction		COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM
DEP	100%	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	+	+++	+
DEP/EtOH	2/98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	0	0	?	0
EtOH	100%	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0
OM	2.0%	+++	++	++	+	++	0	+++	0	0	0	+	0	++	?	+	+	++	?	++	+	+	0	0	0	++	+	++	?	++	0
	1.32%	++	++	+++	+	+	0	++	0	0	0	+	0	++	?	+	+	++	+++	+++	+	++	?	0	0	+++	+	++	0	+++	0
	0.67%	++	+	++	+	+	0	++	0	0	0	0	0	++	?	+	++	++	++	++	+	+	?	0	0	+++	?	+	0	++	0
	0.22%	+++	+	++	?	?	0	++	0	0	0	+	0	+	0	0	0	+	?	++	+	+	+	0	0	++	0	+	+	++	0
	0.074%	+	0	++	?	0	0	+	0	0	0	0	0	+	0	0	0	0	?	++	?	++	?	0	0	+	0	+	?	+++	0
	0.025%	+	0	+	?	0	0	+	0	0	0	0	0	+	0	0	0	++	0	+	0	+	+	0	0	+	0	+	?	+++	+
	0.0082%	?	0	+	?	0	0	?	0	0	0	0	0	0	0	0	0	0	0	+	0	?	?	0	0	+	0	++	++	++	+
	0.0027%	0	0	?	0	0	0	?	0	0	0	0	?	0	0	0	0	0	0	0	0	0	?	0	0	?	0	?	0	0	0
	0.00091%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0
	0.0003%	0	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	?	0	0	0
	0.0001%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	?	0	0	0	0	0	+	?	?
	0.00003%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	+++	0
ROAT		**No. of applications until positive reaction**																													
COM		14		10		-		5		-		-		25		-		39		28		14		-		14		14		5	
NOM		-		33		-		-		-		-		-		-		-		-		-		-		-		28		-	
DEP/EtOH VEH1	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH2	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Amount used		**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**
COM		0.0687	0.9620	0.0549	0.5490	0.0857	3.6000	0.0482	0.2410	0.0925	3.8870	0.0760	3.1940	0.0979	2.4470	0.0808	3.3920	0.0184	0.7160	0.0305	0.8550	0.0403	0.5640	0.0318	1.3370	0.0441	0.6180	0.0466	0.6520	0.0484	0.2420
NOM		0.0781	3.2810	0.0755	2.4920	0.0835	3.5080	0.0458	1.9250	0.0748	3.1420	0.0677	2.8430	0.1290	5.4170	0.0751	3.1560	0.0665	2.7950	0.0782	3.2841	0.0554	3.2250	0.0543	2.2810	0.0553	2.3220	0.0558	1.5620	0.0659	2.7680
DEP/EtOH VEH1	2/98%	0.0619	2.5980	0.1089	4.5750	0.0955	4.0130	0.0474	1.9890	0.0595	2.4970	0.0855	3.5890	0.1031	4.3300	0.1005	4.2200	0.0642	2.6970	0.0817	3.4300	0.1016	4.2670	0.0451	1.8960	0.0596	2.5020	0.0542	2.2750	0.0773	3.2470
DEP/EtOH VEH2	2/98%	0.0857	3.6000	0.0606	2.5440	0.0808	3.3940	0.0532	2.2330	0.0708	2.9720	0.0792	3.3270	0.1442	6.0580	0.0760	3.1930	0.0638	2.6800	0.0679	2.8534	0.0968	4.0640	0.0589	2.4740	0.0495	2.0800	0.0683	2.8680	0.0656	2.7540

Participant		22		26		27		29		39		42		43		46		47		48		50		55		56		60		51	
Original patch test		++		++		++		++		++		++		++		++		++		++		++		++		++		++		+++	
Reaction		COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM
DEP	100%	0	0	0	0	?	0	0	0	0	0	0	0	0	0	++	++	0	0	?	0	++	?	+	0	0	0	+	0	0	?
DEP/EtOH	2/98%	0	0	0	0	0	0	0	0	0	0	0	0	0	?	+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
EtOH	100%	0	0	?	?	0	+	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	?	?	0	0	0	0	0
OM	2.0%	++	?	+	0	+++	?	0	0	+++	0	+++	+	+++	++	+++	++	+	0	+++	+	+++	+++	+++	+	+++	++	+++	+++	+++	+
	1.32%	+	0	+	0	+++	+	0	0	+++	?	++	+	+++	+	+++	+++	+	0	+++	+	+++	+++	+++	+	+++	+	++	+	+++	+
	0.67%	0	0	+	0	+++	+	0	0	++	0	++	?	+++	++	+++	+++	0	0	+++	0	+++	++	+++	+	+++	+	++	+++	+++	?
	0.22%	+	0	+	0	++	0	0	0	++	?	+	?	+++	+	+++	++	0	0	+++	?	+++	?	++	?	++	?	++	+	++	?
	0.074%	0	0	?	0	++	0	0	0	+++	0	+	0	++	+	+++	++	0	0	++	0	+++	+	++	0	++	0	++	+	+++	0
	0.025%	?	0	0	0	+	0	0	0	+++	0	+	?	++	+	+++	++	0	0	++	0	+++	+	++	0	?	0	++	0	++	0
	0.0082%	0	0	0	0	+	0	0	0	0	0	+	0	+	?	++	+	0	0	+	0	+++	+	++	0	?	0	+	?	++	0
	0.0027%	0	0	?	0	+	0	0	0	?	0	0	0	+	0	+++	?	0	0	?	0	+	0	+	0	0	0	+	0	+	0
	0.00091%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	?	0	0	0	?	?	0	0	0	0	0	0	0	?	0
	0.0003%	0	0	0	0	+	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0	?	0	?	0	0	0	?	0	?	0
	0.0001%	0	0	?	0	+	0	0	0	0	0	0	0	0	0	+	0	0	0	0	?	0	0	?	0	0	0	0	0	0	0
	0.00003%	0	0	0	0	0	0	0	0	+	0	0	0	0	0	+	0	0	0	0	0	0	?	0	0	0	0	0	0	0	0
ROAT		**No. of applications until positive reaction**																													
COM		-		14		14		-		28		9		5		14		-		4		5		14		23		6		5	
NOM		-		-		-		-		-		14		28		30		-		-		11		-		-		-		-	
DEP/EtOH VEH1	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH2	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Amount used		**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**
COM		0.0537	2.2560	0.0695	0.9730	0.0711	0.9950	0.0490	2.0590	0.0314	0.8780	0.0986	0.8870	0.0728	0.3640	0.0597	0.8360	0.0378	1.5880	0.0610	0.2440	0.0764	0.3820	0.0714	0.9990	0.0675	1.5520	0.0612	0.3670	0.0558	0.2790
NOM		0.0478	2.0060	0.0911	3.8250	0.0804	3.3760	0.0439	1.8430	0.0843	3.5410	0.0909	1.2730	0.0489	1.3700	0.0943	2.8290	0.0297	1.2460	0.0371	1.5570	1.0540	1.0540	0.0727	3.0540	0.0705	2.9620	0.0523	2.1970	0.0365	1.5310
DEP/EtOH VEH1	2/98%	0.0482	2.0250	0.0676	2.8390	0.0580	2.4340	0.0697	2.9290	0.1093	4.5900	0.1031	4.3290	0.0452	1.8980	0.0844	3.5460	0.0319	1.3410	0.0606	2.5460	0.1120	4.7050	0.0585	2.4590	0.0560	2.3520	0.0501	2.1050	0.0352	1.4790
DEP/EtOH VEH2	2/98%	0.0573	2.4060	0.0752	3.1570	0.1090	4.5800	0.0527	2.2120	0.0844	3.5440	0.0713	2.9940	0.0456	1.9150	0.0823	3.4580	0.0320	1.3450	0.0525	2.2070	0.1175	4.9340	0.0727	3.0520	0.0525	2.2050	0.0410	1.7240	0.0372	1.5610

COM, classic oak moss; DEP, diethyl phthalate; EtOH, ethanol; NOM, new oak moss; OM, oak moss; PA, per application; ROAT, repeated open application test; VEH, vehicle.

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Table 2. Raw data for control subjects in inclusion order

Participant		1		2		6		7		8		9		10		12		13		14		16		17		18		20		23	
Original patch test		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Reaction		COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM
DEP	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DEP/EtOH	2/98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EtOH	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
OM	2.0%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	0	0
	1.32%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0
	0.67%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.22%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.074%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
	0.025%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.0082%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
	0.0027%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00091%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?
	0.0003%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.0001%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00003%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ROAT		**No. of applications until positive reaction**																													
COM		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
NOM		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH1	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH2	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Amount used		**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**
COM		0.0658	2.7620	0.1166	4.8980	0.0257	0.7190	0.1043	4.3800	0.0933	3.9200	0.0841	3.5330	0.0529	2.2230	0.0627	2.6340	0.2338	0.9350	0.0402	1.6870	0.2072	1.0360	0.0659	2.7670	0.1178	0.5890	0.0318	1.3370	0.0331	1.3900
NOM		0.0610	2.5610	0.1042	4.3770	0.0317	1.3320	0.1069	4.4910	0.0779	3.2700	0.0805	3.3800	0.0482	2.0240	0.0469	1.9700	0.0585	2.4570	0.0475	1.9960	0.0858	3.6030	0.0980	4.1150	0.0480	2.0180	0.0461	1.9350	0.0310	1.3030
DEP/EtOH VEH1	2/98%	0.0719	3.0200	0.0719	3.0200	0.0212	0.8920	0.1243	5.2190	0.0920	3.8660	0.0708	2.9740	0.0486	2.0410	0.0427	1.7930	0.0522	2.1920	0.0406	1.7040	0.0847	3.5570	0.0706	2.9670	0.0695	2.9170	0.0507	2.1310	0.0370	1.5540
DEP/EtOH VEH2	2/98%	0.0742	3.1170	0.0754	3.1670	0.0396	1.6650	0.0928	3.8960	0.0806	3.3870	0.0734	3.0840	0.0432	1.8150	0.0461	1.9380	0.0435	1.8290	0.0374	1.5720	0.0737	3.0970	0.0991	4.1640	0.0525	2.2070	0.0417	1.7530	0.0339	1.4220

Participant		24		25		31		34		38		40		41		45		49		52		53		54		57		58		59	
Original patch test		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Reaction		COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM	COM	NOM
DEP	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DEP/EtOH	2/98%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EtOH	100%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OM	2.0%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1.32%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0
	0.67%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0
	0.22%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0
	0.074%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.025%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0
	0.0082%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.0027%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00091%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.0003%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.0001%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00003%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ROAT		**No. of applications until positive reaction**																													
COM		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
NOM		-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH1	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
DEP/EtOH VEH2	2/98%	-		-		-		-		-		-		-		-		-		-		-		-		-		-		-	
Amount used		**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**	**PA**	**Total**
COM		0.1564	0.7820	0.0270	0.3780	0.0277	1.1630	0.0586	2.4628	0.0762	3.2010	0.0844	0.7600	0.0426	0.9790	0.0485	2.0380	0.1025	0.6150	0.0326	0.4570	0.0853	3.3270	0.0292	1.2250	0.0712	2.9890	0.0511	0.5110	0.0470	1.9720
NOM		0.0635	2.6660	0.0263	1.1030	0.0314	1.3190	0.0542	2.2780	0.0935	3.9270	0.0819	3.4390	0.0432	1.8140	0.0471	1.9800	0.0432	1.8130	0.0237	0.9970	0.0705	2.9620	0.0286	1.2030	0.0493	2.0690	0.0266	1.1190	0.0537	2.2570
DEP/EtOH VEH1	2/98%	0.0751	3.1530	0.0494	2.0750	0.0323	1.3570	0.0501	2.1030	0.1115	4.6840	0.0570	2.3960	0.0404	1.6950	0.0378	1.5860	0.0459	1.9270	0.0275	1.1570	0.0627	2.6340	0.0338	1.4200	0.0629	2.6430	0.0316	1.3270	0.0574	2.4100
DEP/EtOH VEH2	2/98%	0.0820	3.4420	0.0474	1.9913	0.0589	2.4740	0.0276	1.1590	0.0501	2.1060	0.0832	3.4950	0.0872	3.6620	0.0349	1.4640	0.0465	1.9520	0.0256	1.0770	0.0574	2.4100	0.0268	1.1270	0.0599	2.5150	0.0297	1.2470	0.0535	2.2470

COM, classic oak moss; DEP, diethyl phthalate; EtOH, ethanol; NOM, new oak moss; OM, oak moss; PA, per application; ROAT, repeated open application test; VEH, vehicle.

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in the control group. The mean age at the time of inclusion was 51.2 years [standard deviation (SD): ±12 years] for the oak moss-sensitive group and 48.8 years (SD: ±10.3 years) for the control group. On average, oak moss-sensitive subjects had a previous positive patch test reaction to oak moss at a level of 1% or 2% in petrolatum ~5.7 years prior to the start of the study.

In each of the two groups, 7 subjects reported having had childhood eczema; in 13 of these, the diagnosis had been confirmed by a doctor. Nearly half of the test subjects experienced rhinitis caused by airborne allergens (oak moss-sensitive group, n = 15; control group, n = 13). Fourteen subjects from the oak moss-sensitive group and 11 subjects from the control group had shown positive blood and/or prick test reactions. Grass and birch pollen, dust mite and animal allergens were the most common causes of allergies.

Two oak moss-sensitive subjects and 6 control subjects had been diagnosed with asthma, and 9 oak moss-sensitive subjects and 7 control subjects had hay fever.

Four oak moss-sensitive subjects claimed to have experienced hives caused by food and animals, and 5 to have experienced hives after contact with latex. In the control group, 6 subjects reported having experienced hives caused by the above.

Twenty-one of 30 oak moss-sensitive subjects and 17 of 30 control subjects had family members with hay fever, asthma, or childhood eczema.

Approximately two-thirds of oak moss-sensitive subjects reported allergy or sensitivity to perfumes, toilet waters or other highly perfumed products (n = 23), skin creams and/or lotions (n = 19), and deodorants or antiperspirants (n = 22). Approximately one-third of oak moss-sensitive subjects (n = 10) reported allergy or sensitivity to products to be washed off the skin, such as bar soaps and waterless hand-cleansers. In contrast, only a few control subjects (n ≤2) reported being sensitive to the above.

Approximately two-thirds (n = 23) of oak moss-sensitive subjects reported hypersensitivity reactions to clothing, jewellery/metals, and bandages or tape, whereas approximately one-third (n = 12) of control subjects had hypersensitivity reactions to the same lines of product.

Fewer than one-third of oak moss-sensitive subjects reported being sensitive to the following products: men's cologne and aftershaves (n = 8), hair care products (n = 7), eye make-up products (n = 8), other cosmetic products (n = 4), bath oils, salts, beads (n = 4), shampoos, cream rinses, conditioners (n = 8), shaving products (n = 6), laundry detergents, softeners (n = 9), liquid dishwashing detergents (n = 6), scouring powders, hard surface cleansers (n = 7), air fresheners (n = 3), and mouthwashes or toothpastes (n = 2). Again, only a couple of control subjects (n ≤4) reported being sensitive to the same products.

Phase I ROAT

No control subjects reacted to either solution of oak moss; neither control subjects nor known oak moss-sensitive subjects had positive reactions to the vehicles.

Twenty-two of 30 oak moss-sensitive subjects had a positive reaction to COM; of these, only 6 reacted to NOM. No subjects reacted to NOM only. McNemar's test with the continuity correction showed that COM caused significantly more reactions than NOM ($p = 0.0002$).

Among the subjects who had a positive ROAT result, the mean numbers of applications needed to elicit a positive reaction were 15 for COM and 26 for NOM ($p < 0.0001$, Wilcoxon's test). The total amount of test solution with COM required to elicit a positive ROAT result in the most sensitive subject was 10 mg/cm^2, and the average for the 22 subjects with positive ROAT results was 30 mg/cm^2. For NOM, the most sensitive subject reacted to 55 mg/cm^2, and the average for the 6 subjects with positive ROAT results was 71 mg/cm^2 (Fig. 1)

The same amount of each test substance was applied at each application, regardless of the sample type (COM, NOM, or vehicle) (Friedmann test with Dunn's multiple comparisons test, $p = 0.5207$).

In total, significantly less COM than both NOM and vehicle was applied. There was no difference between the amount of NOM and the amount of vehicle applied (Friedmann test with Dunn's multiple comparisons test, $p < 0.0001$). Removing the subjects who reacted to DEP in the patch test did not alter the outcome of statistical tests.

Phase II patch test

None of the control subjects had a positive reaction to either solution of oak moss (Table 2). Three subjects with previous positive patch test reactions to oak moss did not respond to oak moss absolute in either the ROAT or the patch test. Two subjects who had reacted in the ROAT did not react in the patch test. One subject reacted to the same concentrations of the two different oak moss preparations.

Among the oak moss-sensitized subjects, 3 had positive reactions to DEP in both patch test blocks (subjects 21, 46, and 50); in subject 46, it was not possible to distinguish the reaction caused by DEP from those caused by COM and NOM.

McNemar's test with the continuity correction showed that COM caused significantly more reactions than NOM ($p = 0.0077$). The difference remained significant after

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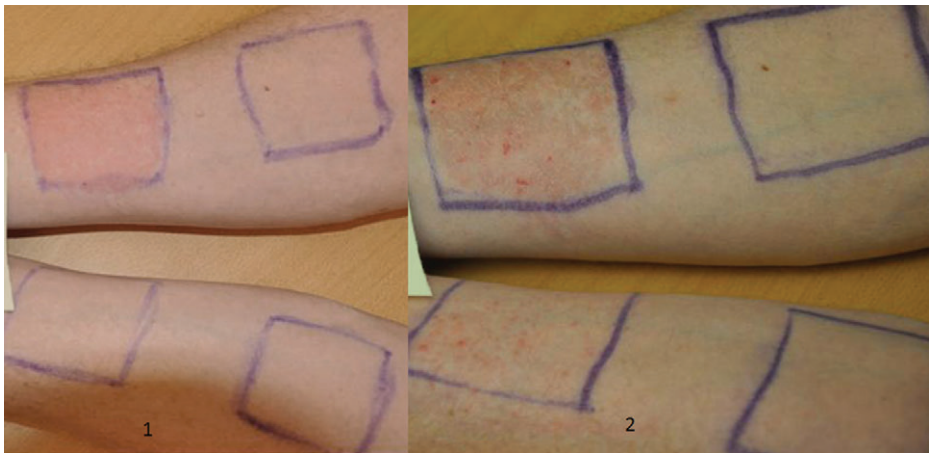

Fig. 1. Positive repeated open application test result in subject 50. Image 1 demonstrates a confluent reaction to classic oak moss absolute on application no. 5. Image 2 demonstrates a spotty reaction to new oak moss absolute on application no. 11.

removal of the subjects who reacted to DEP in the patch test ($p = 0.0133$).

To evaluate the difference in concentration necessary to elicit an allergic response, Wilcoxon's test was performed, after removal of the above-mentioned 6 subjects from the calculation. The remaining subjects reacted to a significantly lower concentration of COM than of NOM (Wilcoxon's test, $p < 0.0001$).

After removal of subjects for whom the interpretation regarding NOM readings could be challenged (subjects 5, 19, 21, 22, 35, 36, and 39), there was still a significant difference in the concentration eliciting a positive reaction (Wilcoxon's Test $p < 0.0001$), but no difference in the number of positive reactions was observed with McNemar's test with the continuity correction ($p = 0.2482$).

Correlations

In a graphical comparison of the dose–response relationships for the two oak moss preparations, it appeared that NOM behaved almost like a diluted version of COM (Fig. 2).

The correlation between the number of applications until a positive ROAT result and the threshold patch test concentration is shown in Fig. 3. The relationship was significant with regard to COM ($p = 0.0475$; Spearman rank = 0.4372), but there were too few data points for NOM for such a calculation to be performed.

The correlation between the number of applications until a positive ROAT result and the total amount applied (based on weighing of dosing containers before and after use) until a positive reaction was extremely significant with regard to COM ($p = 0.0007$; Spearman rank = 0.6794), but there were too few data points for NOM for such a calculation to be performed.

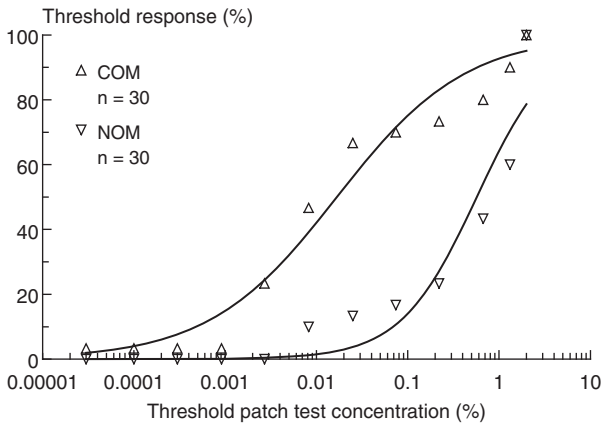

Fig. 2. Dose–response curves derived as accumulated distributions of threshold reactions to classic oak moss absolute (COM) and new oak moss absolute with lowered contents of atranol and chloroatranol (NOM) based on paired data from 30 oak moss-sensitized subjects.

Discussion

The chemical composition of the oak moss extracts is very complex, as they contain > 170 substances. Two of these substances, atranol and chloroatranol, are the most important sensitizers in oak moss absolute (2, 3).

In a murine model, it has been shown that the mixture of allergens in oak moss absolute is more potent than atranol and chloroatranol alone (15), but reducing the contents of the two major allergens should still result in a less potent allergen mixture.

The selective removal of atranol and chloroatranol without significantly affecting the olfactory properties of moss extracts is a challenge for industrial

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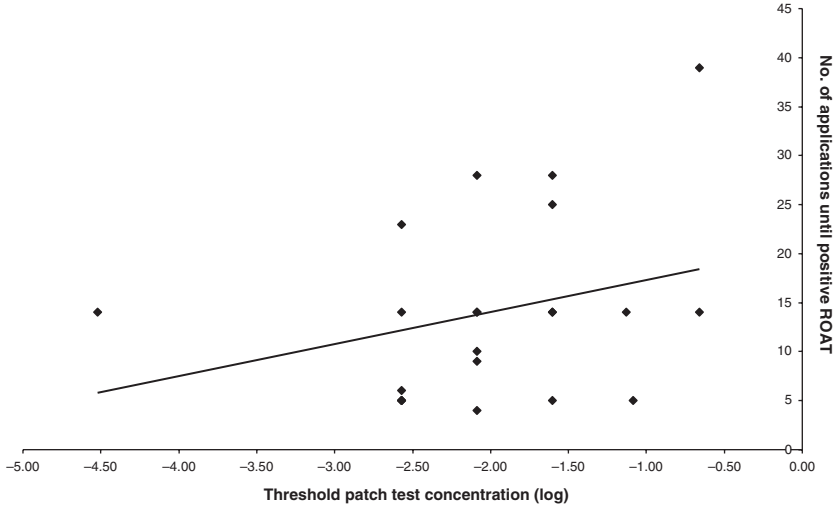

Fig. 3. The correlation between the number of applications until a positive repeated open application test (ROAT) result and the threshold patch test concentration (log-transformed). The relationship was significant with regard to classic oak moss absolute ($p = 0.0475$; Spearman rank = 0.4372), but there were too few data points for new oak moss absolute for such a calculation to be performed.

production. However, it appears to have been satisfactorily accomplished, as perfumers state: 'The low atranol/chloroatranol quality is less marine/seaweed/kelp/iodine and softer resin/balmy/kind citrus but overall one recognizes well the character of the regular Oak Moss extract' (pers. comm. from G Robertet to FA, 2013).

Because of the unavoidable variability in an industrial process, manufacturers of oak moss absolute can only guarantee < 100 ppm each of atranol and chloroatranol after reduction.

In this study, subjects were stratified adequately with regard to age and sex. The reported sensitivities to common irritants and allergens in the oak moss-sensitive group and the control group were as expected, as was the relatively high prevalence of atopy among both groups.

A time period of 3 weeks ensured that we could readily distinguish between the two closely related oak moss preparations. We do not know whether more applications of NOM would reduce the difference.

In the ROAT, the subjects applied the same amount of each test substance at each application, but significantly less COM was used in total, as expected because COM applications were terminated earlier than applications of the other test solutions. This was supported by the findings that significantly more subjects reacted to COM than to NOM, and that significantly fewer applications of COM than of NOM were necessary to elicit a positive reaction.

Three of 30 subjects for whom sensitization to oak moss had been diagnosed in the past did not react to either oak moss formulation. 'Losing' a contact allergy over time is a well-known phenomenon (16).

DEP is used as a safe vehicle constituent in experimental dermatology, both in studies as described herein and in the local lymph node assay (17). Furthermore, DEP is used as a plasticizer in hard clear cellulose ester plastics, which are used to manufacture products with prolonged skin contact, such as hearing aids and frames for spectacles. In plastics, DEP has been described as a rare sensitizer. DEP may cause both irritant and allergic contact dermatitis (18). In the study described, herein 3 of 60 subjects, all oak moss-sensitive, showed a positive reaction to neat DEP in the patch test. The positive reactions may be attributable to neat DEP being a mild irritant. The recommended patch test concentration according to de Groot is 2–10% pet. (19). Removing these 3 subjects from the calculations did not alter the findings.

NOM differed significantly from COM with regard to its ability to elicit an allergic response, and thus most likely in its ability to induce allergy. The correlation between threshold patch test concentration and number of days until a positive ROAT result was less evident than in a similar study on isoeugenol (9). This may be attributable to the nature of oak moss as a natural extract with many components, meaning that the pattern of allergy may vary between individuals. In contrast, isoeugenol is a well-defined chemical.

As shown in the dose–response curves (Fig. 2), the findings reported herein confirm and amplify the findings in the pilot study, showing that reactivity to NOM is significantly lower than reactivity to COM, and that NOM is less likely to induce sensitivity than COM (7).

Conclusions

NOM elicited significantly less allergic contact dermatitis in previously oak moss-sensitive subjects in a human experimental exposure model than COM.

NOM still has to be regarded as a fragrance allergen, but, on the basis of the dose–response curve, is a distinctively weaker allergen than COM.

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The fact that NOM elicits less allergic contact dermatitis in previously oak moss-sensitized individuals suggests that NOM also is less allergenic for use in non-sensitized individuals.

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