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Are atranols the only skin sensitizers in oakmoss? A systematic investigation using non-animal methods

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ABSTRACT

Oakmoss and treemoss absolutes are the major natural extracts of concern as potential sources of skin sensitizers in cosmetics and personal care products (PCP). Two single constituents, atranol and chloroatranol, have been identified as primary culprits in both lichens, and industrial self-regulation has been proposed to limit their contents to less than 100 ppm. Nonetheless, evidence points to the presence of additional candidate skin sensitizers in these multicomponent extracts. These observations, along with a lack of data from non-animal alternative methods and the chemical variability of commercial absolutes, prompted further investigation of oakmoss absolute along with altranol-like compounds in these extracts. The major chemical constituents of a commercial sample were identified by two independent analytical techniques, GC–MS and HPLC-DAD-MS. The crude oakmoss extract and pure compounds were assayed with two *in chemico* methods (HTS-DCYA and DPRA) to gauge their chemical reactivity. Activation of inflammatory responses *in vitro* was also investigated by KeratinoSens™ and human cell line activation tests (h-CLAT). Based on weight of evidence, orcinol, ethyl orsellinate, and usnic acid were classified as candidate sensitizers, along with both atranols and oakmoss extract.

1. Introduction

Oakmoss absolute (OMA) is one of the most popular natural fragrance ingredients with a characteristic woody aroma and fixative properties. Oakmoss is a core component of the fragrance family renowned as '*Chypre accord*', a classic scent often related to high-end, luxurious products including some of the most iconic perfumes. (Kirk-Othmer, 2012) Oakmoss absolute is obtained from the lichen *Evernia prunastri* var. *prunastri* (L.) ch. (Parmeliaceae), grown in the south-central regions of Europe as well as in Morocco and Algeria. The production of OMA has been evolving over the years, as the use of carcinogenic solvents (*viz.*, benzene) have been progressively abandoned. Nowadays, the lichen is typically desiccated and pre-humidified before extraction with either hexane or mixtures of hexane and other solvents, mainly acetates. This aqueous pre-treatment can be severe, sometimes involving high temperatures and humidity. Each commercial manufacturer has optimized its exclusive processes to obtain proprietary blends. Therefore, processing conditions and chemical composition of commercially available oakmoss ingredients may vary considerably. The resulting extracts (resinoids) are often subjected to alcohol treatment to obtain OMA (or 'soluble resinoids'). Typically, OMA contains a mixture of β-resorcinol derivatives, which are the hydrolytic and/or decarboxylated products of depsides.(Ter Heide et al., 1975; Schulz and Albroscheit, 1989) These hydrolytic products are responsible for the

Abbreviations: AOP, adverse outcome pathway; ATR, atranol; ATN, atranorin; CINN, cinnamaldehyde; ClATR, chloroatranol; DCYA, dansyl cysteamine; DPRA, direct peptide reactivity assay; EE, ethyl evernitate; EO, ethyl orsellinate; FM I, Fragrance mix I; GC–MS, Gas chromatography coupled with mass spectrometry; h-CLAT, human cell line activation test; HPLC-PDA-MS, high performance liquid chromatography coupled with diode array detector and mass detector; HTS, high throughput screening; ICH, isopropyl chlorohaematommate; IE, isopropyl evernitate; IFRA, International Fragrance Association; IH, isopropyl haematommate; KE, key event; KS, KeratinoSens™; ITS, integrated testing strategy; MA, methyl atratate; MIE, molecular initiating event; MMP, 3-methoxy-5-methylphenol; OECD, Organisation for Economic Co-operation and Development; ORC, orcinol; β-ORC, β-orcinol; OMA, oakmoss absolute; NMR, nuclear magnetic resonance; RI, reactivity index; UA, usnic acid.

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characteristic 'mossy' fragrance of OMA. When a solvent like ethanol is used for extracting the lichen, direct trans-esterification of β-resorcinoids can occur during the later processing of the resinoid to the absolute. The absolute can be further processed to meet the desired features such as avoiding the original dark brown color with discoloration or high vacuum distillation. The intrinsic chemical variability associated with this lichen can thus be aggravated by the type and conditions of treatment used, thus making it difficult to associate oakmoss composition (and its olfactory properties) to a specific chemical fingerprint. (Joulain and Tabacchi, 2009a).

Recent regulatory history has placed this highly popular fragrance ingredient under the spotlight due to safety concerns associated with topical allergic reactions. Oakmoss absolute is one of the ingredients of the fragrance mix I (FM I) used in the European baseline standard patch test series (Larsen, 1977), and untreated oakmoss is considered unsafe for consumers. (SCCP, 2008).

The aldehydes, atranol (ATR) and chloroatranol (ClATR) have been identified as the main skin allergens in oakmoss. (Bernard et al., 2003) The two compounds are the hydrolytic products of the odorless precursor depsides, atranorin (ATN) and chloroatranorin, respectively. ClATR has been classified as a more potent allergen than ATR, but exposure to ATR in commercial oakmoss products is greater than its chlorinated analog. (Rastogi et al., 2004; Johansen et al., 2006) The development of hypo-allergenic formulations, without altering the typical organoleptic character of oakmoss, has been the object of many studies. Pre-treatment of OMA to remove the aldehydes can be achieved by either chromatographic techniques, solvent extraction, through soluble amino acids, insoluble polymer scavenging, catalytic hydrogenation, or alkaline treatment. These processes may lack selectivity for atranols, as other aromatic aldehydes and depsides present in the absolute can be removed as well. (Ehret et al., 1992) Repeated open application test and serial dilutions patch tests performed on human volunteers demonstrated that OMA treated to lower the ATR/ClATR concentration to less than 50 ppm was still allergenic, although the potency was significantly lowered. (Mowitz et al., 2014; Andersen et al., 2015) A self-regulatory restriction on ATR content has been recommended by the International Fragrance Association (IFRA), which limits the content of ATR and ClATR to less than 100 ppm each. (Anon, 2009).

Although atranols skin sensitization potential has been repeatedly demonstrated, further evidence points toward the presence of multiple skin sensitizers in addition to the atranols. Atranorin, methyl- and ethyl chloro-haematommate, methyl atratate (MA), evernic acid, fumaroprotocetraric acid and usnic acid (UA) have been identified as potential sensitizers in patch test studies. (Dahlquist and Fregert, 1980; Thune et al., 1982; Sandberg and Thune, 1984; Gonçalo et al., 1988; Bernard et al., 2003) Moreover, patch tests with thin layer chromatography (TLC) strips of OMA samples *vs.* OMA with low atranols indicated the presence of at least ten compounds capable of producing allergic responses, and low atranols preparations (total conc. 66 ppm) still resulted in positive reactions. (Mowitz et al., 2013) The majority of oakmoss constituents include a β-resorcinol functional group which can be considered as a pre/pro-hapten and additional aldehyde functional group can also be classified as haptens, due to their ability to form Schiff's bases with hard nucleophiles (amines or amino acids).

The scope of the present work was to investigate the skin sensitization potential of main atranols and atranol-like oakmoss costituents using a combination of chemical and *in vitro* methods to gain a better understanding of the mechanism of action of oakmoss and its constituents on the molecular initiating event (MIE) leading to skin sensitization and further biological pathways, as outlined by the adverse outcome pathway (AOP) paradigm. (Vinken, 2013) To this end, a commercial oakmoss absolute sample was characterized using two independent analytical methods to identify the main constituents.(Chittiboyina et al., 2020) Unknown compounds were isolated and their identity was established *via* nuclear magnetic resonance (NMR) and GC–MS experiments. To gauge the sensitization potential of the main constituents of OMA, a combination of four non-animal methods was investigated. Two *in chemico* methods, the high-throughput DCYA method (HTS-DCYA) and the Direct Peptide Reactivity Assay (DPRA) were used to characterize the chemical reactivity of oakmoss absolute constituents. Such chemical reactivity is considered relevant to promote the MIE. The second Key Event (KE 2), the elicitation of the inflammatory pathway in keratinocytes, was characterized using the KeratinoSens™ (KS) method. The human Cell Line Activation Test (h-CLAT) was performed to characterize the activation of stress responses in dendritic cells (KE 3).

2. Material and methods

Caution:*All tested compounds may cause skin allergy. These compounds must be handled with care.*

2.1. Chemicals and reagents

The oakmoss absolute (NCNPR # 18333) was obtained from Vanda Rose, Hebden Bridge HX7-5TT, UK (prod. N. B.01E.1812). Cinnamaldehyde (CINN, CAS N. 14371–10-9), ethyl orsellinate (EO, CAS 2524-37-0), methyl atratate (MA, CAS 4707-47-5), 3-methoxy-5-methylphenol (MMP, CAS 3209-13-0), orcinol (ORC, CAS 504–15-4), β-orcinol (β-ORC, CAS 488–87-9), usnic acid (UA, CAS 7562-61-0), coumarin (COUM, CAS 91–64-5), HPLC grade acetone (>99.9%) and Sephadex® LH-20 (GE Healthcare, 17–0090) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ATN (479–20-9), ATR (CAS 526–37-4) and ClATR (CAS 57074–21-2) were purchased from Carbosynth Ltd. (Compton, Berkshire, UK). Flash column chromatography purifications were performed on Biotage Isolera Four, using SNAP cartridges (Biotage, LLC, Charlotte, NC USA). Silica gel and reversed-phase cartridges were KP-Sil and KP-C18-HS, respectively.

Cysteine peptide (Ac-RFAACAA-COOH, purity 94.9%, cat. N. RSC998) and lysine peptide (Ac-RFAAKAA-COOH, purity 91.1%, cat. N. RSC999) were purchased from RS Synthesis (Louisville, KY, USA). Sodium phosphate buffer (Na_2HPO_4 / NaH_2PO_4, 100 mM pH 7.4) was used for Cys-DPRA reactions; ammonium acetate buffer (CH_3COONH_4/ NH_4OH, 100 mM pH 10.2) was used for Lys-DPRA reactions.

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 96-well microplates, isolation solvents and HPLC grade water and acetonitrile were purchased from Fisher Scientific (Suwanee, GA, USA).

2.1.1. Isolation of ethyl everninate (EE), isopropyl everninate (IE), isopropyl haematommate (IH), and isopropyl chlorohaematommate (ICH)

Commercial OMA sample CGE-35 (2.63 g) was dissolved in equal parts of acetone/CH_2Cl_2 and loaded on a 100 g Sephadex LH-20 column (2 × 60 mm) pre-equilibrated with acetone/CH_2Cl_2 (1:1) and eluted at 0.4 mL/min. The fractions containing the compounds of interest were combined (670 mg total) and resolved first on silica gel eluted with hexane/diethyl ether (98/2), followed by RP-C18 purification using a gradient phase H_2O/MeOH from 50 to 80% of MeOH. Compounds thus obtained were identified by NMR and GC–MS.(Chittiboyina et al., 2020).

2.1.2. Analytical characterization of oakmoss absolute

Gas chromatographic analyses were performed using an Agilent 7890A GC system equipped with a 5975C quadrupole mass spectrometer and a 7693 autosampler (Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was performed on a DB-5MS capillary column (Agilent J&W Scientific, Folsom, CA, USA) with a dimension of 30 m × 0.25 mm i.d. × 0.25 μm film thickness. Helium was used as the carrier gas at a flow rate of 1 mL/min. The inlet temperature was set to 280 °C with a split injection mode for a split ratio of 15:1. The oven

temperature program was as follows: the initial temperature was 100 °C (held for 2 min), increased to 160 °C at a rate of 4.0 °C /min (held for 4 min), then to 280 °C at a rate of 8 °C /min, and isothermal for 8 min at 280 °C with a total experiment time of 44 min. Mass spectra were recorded at 70 eV at a scan mode from *m/z* 35 to 500. The transfer line temperature was 280 °C. The ion source and quadrupole temperatures were 230 and 130 °C, respectively. Data acquisition was performed with Agilent MSD Chemstation (F.01.03.2357).

High performance liquid chromatographic analyses were performed using an Agilent 1290 Infinity HPLC system equipped with a quaternary pump, autosampler, column compartment, photodiode array detector, and Agilent Open Lab ChemStation data acquisition software (Agilent Technologies, Santa Clara, CA, USA). An SB-C18 RRHD, 2.1 × 100mm × 1.8 μm UPLC column was used (Agilent Technologies). The flow rate was adjusted to 0.200 mL/min. The mobile phase system used was composed of water +0.2% (*v/v*) acetic acid (phase A) and acetonitrile +0.2% (*v/v*) acetic acid (phase B). Gradient elution was performed at 30 °C by linear gradient from 35 to 55% of solvent B over 4 min, then linear increase to 65% B in 16 min, then from 65 to 95% in 2 min. Positive and negative ESI mass range detector set up was between *m/z* 100 and 1000, fragmentor was set up to 70 eV.

2.2. HTS-DCYA

The chemical reactivity was assessed using the *in chemico* HTS-DCYA method as previously described (Avonto et al., 2015; Avonto et al., 2018) by mixing equal volumes of 2.5 mM DCYA in acetonitrile with 5 mM of test article. The reaction was started by addition of pH 10 buffer for 24 min, and stopped by incubation of a DCYA scavenger for 45 min. The degree of reactivity is reported as the Reactivity Index (RI), corresponding to the estimated formation of covalent DCYA adducts with the test article. The RI was calculated based on the average of nine readings per sample (three readings/well in triplicate) as previously reported. CINN and COUM were used as positive and negative control, respectively.

The fluorescent readings were acquired on a SpectraMax M5 multimode 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. DPRA

The DPRA method was performed as described by the Organisation for Economic Co-operation and Development guidelines (OECD, 2020). Acetonitrile HPLC grade was used to dissolve the test articles. A Zorbax SB-C-18 3.5 μm HPLC column (2.1 mm × 100 mm) was used and analyses were performed using the Agilent 1290 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA). The flow rate was adjusted to 0.350 mL/min, and gradient elution set as follows: linear gradient from 10 to 25% of solvent B over 10 min, followed by increase to 90% B in 1 min, then from 90 to 100% in 1 min, and hold for 7 min before column re-equilibration (10 min). Absorbance at 220 nm was used to determine the peptide depletion. A calibration curve for each peptide was calculated from 0.0156 to 0.5 mM. Reaction controls were performed using CINN (positive control) or solvent (negative control). Average results of three independent reactions were taken. Negative percentages of depletion were considered as a '0' depletion and averaged accordingly. The samples were analyzed and classified according to the cysteine 1:10/lysine 1:50 prediction model (threshold 6.38%) or to the cysteine 1:10 prediction model when co-elution with the lysine peptide was found (threshold 13.89%).OECD, 2020.

2.4. KeratinoSens™ assay

The assay was performed using KeratinoSens™ cell line which was obtained from Givaudan (Vernier, Switzerland). The cells were cultured in DMEM supplemented with GLUTAMAX, FBS (10%), and G418 (500 μg/mL) at 37 °C, in an atmosphere of 5% CO_2 and 95% humidity. Upon reaching a confluency of 80–90%, the cells were seeded in 96-well white plates (10,000 cells/well) for the luciferase assay. Cells were also seeded in clear 96-well plates for the MTT assay. After incubating for 24 h, the medium was replaced with antibiotic free medium containing 1% FBS. Prior to the assay, samples were dissolved in DMSO (200 mM) and diluted in 1% FBS antibiotic free medium before adding to the cells. Cells were treated with test samples for 48 h. At the end of the incubation period, cells were washed with calcium and magnesium free DPBS and incubated with lysis buffer for 20 min at room temperature. Promega firefly luciferase reagent was added and the luminescence was immediately measured on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, California, USA). Increase in luciferase activity in sample treated cells was calculated in comparison to DMSO treated cells (negative control). To determine the cell viability under similar experimental conditions, media was removed from the clear 96-well plate and cells were incubated with a solution of MTT (0.5 mg/mL in serum free media) for 4 h at 37 °C. The media was removed and DMSO was added to each well to dissolve the blue formazan produced by the cells, the color of which was read at 570 nm on a SpectraMax M5 plate reader. Positive control was CINN, while DMSO (1%) was the negative control.

Efficacy of test samples was calculated in terms of EC1.5 which was calculated using the following equation (ECVAM, 2014; OECD, 2018a):

$$EC1.5 = (C_b – C_a) \times (1.5 - l_a/I_b - I_a) + C_a$$

EC1.5 is defined as the concentration that causes an increase of 1.5 fold in luciferase activity, where C_a is the concentration above 1.5 fold induction; C_b is the concentration below 1.5 fold induction; I_a is the fold induction above 1.5 fold induction and I_b is the fold induction below 1.5 fold induction.

2.5. H-CLAT

Human cell line activation assay was performed in THP-1 cells (obtained from ATCC, Manassas, VA). The cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 0.05 mM 2-mercaptoethanol and 1% antibiotic-antimycotic (Fisher Scientific). Test chemicals were dissolved in DMSO (250 mg/mL). The final DMSO concentration in the assay medium did not exceed 0.2%. For cytotoxicity testing prior to activation assay, THP-1 cells were added to the wells of 24-well plates (1×10^6 cells per mL per well) and exposed to various concentrations of a test chemical (500 μg/mL being the highest concentration). The cells were then washed twice with FACS buffer (DPBS with 0.1% BSA), stained with propidium iodide and analyzed by flow cytometry, using a FACSCalibur equipped with a CELLQUEST software. The concentration of the test chemical that kept 75% cell viability, termed CV75, was calculated based on the analysis of viable cells. For the cell activation assay, THP-1 cells (1×10^6 cells mL^{-1} per well in 24-well plates) were incubated for 24 h with various concentrations of test sample, with the highest concentration being 1.2 x CV75. Following exposure, the cells were first washed with FACS buffer then resuspended and washed with a blocking buffer containing 0.01% globulins Cohn fraction II/III (Sigma-Aldrich). Cells were then incubated for 30 min at 4 °C with the following monoclonal antibodies: APC mouse IgG1 (clone: MOPC-21) from BD Pharmingen, mouse anti-human CD54, ICAM-1/FITC (clone: 6.5B5) from Dako, and FITC mouse anti-human CD86 (clone: 2331 FUN-1) from BD Pharmingen. The cells were washed and stained with propidium iodide and the fluorescence intensity of the viable cells was analyzed using the FACSCalibur. The relative fluorescence intensities (RFIs) of CD86 and CD54 were calculated. If the RFI of CD86 or CD54 was greater than 150 or 200%, respectively, at any dose in at least two experiments, the test chemical was judged as a sensitizer. DNCB (1-chloro-2,4-

dinitrobenzene, Sigma Aldrich) was included as a positive control in each experiment. (OECD, 2018b)

3. Results

Oakmoss absolutes are mainly composed of a variable mixture of resorcinol-like compounds, including orcinols, orsellinic acid esters, often containing aromatic aldehydes and chlorinated moieties as potential mechanistic domains of concern for skin sensitization.(Aptula et al., 2005; Basketter et al., 2007) The exact composition will vary based on the manufacturer's processing among other variables such as edaphic conditions, harvesting methods, and external contaminations.

3.1. Chemical composition of OMA

The commercial OMA here analyzed was a dark-brown gum with an intense aroma. The chemical fingerprinting of the sample (obtained by both GC–MS and HPLC-MS, Figs. 1 and 2) was characterized by 12 main constituents (Table 1). Comparison with commercial reference standards enabled the rapid identification of about 88.9% of the composition of the sample. The main peaks in the GC–MS chromatogram were identified as MMP, ORC, and MA by comparison with reference standards (Fig. 3); contributing to about 47.5% of the total composition based on peak area. ATR and ClATR were found at concentrations of 7.35 and 3.59% (area %), respectively. Although no information is available on the processing method for OMA, the abundance of the atranols, along with several aromatic aldehydes, supports speculation that the studied sample was not subjected to any treatment for the selective removal of atranols. The ratio ATR:ClATR was about 2:1, which is consistent with previously reported findings. (Bernard et al., 2003). Usnic acid, a non-hydrolyzable depside, was the only dimeric compound identifiable in significant amount (1.62%), while no quantifiable amount of ATN or chloroatranorin were identified. The atranorin standard was detectable in the developed HPLC-MS method, but again, only trace amounts of atranorin were found in the commercial sample studied.

The remaining major unidentified peaks in the GC–MS chromatogram had *m/z* values of 210, 224, 224, 238 and 272, respectively. An aliquot of the OMA sample was thus subjected to purification to isolate the unknown compounds. The pure compounds isolated were identified by NMR spectroscopy as ethyl everninate, isopropyl everninate, ethyl haematommate, isopropyl haematommate and isopropyl chlorohaematommate. (Chittiboyina et al., 2020) Isopropyl derivatives were detected in this sample in unusually high amounts. Isopropyl haematommate could be obtained as extraction artifact from ATN, and has been reported in the case of treemoss. (Joulain and Tabacchi, 2009b) Substitution with tree moss is unlikely due to the undetectable levels of olivetonide in the volatile fraction (expected *m/z* 248), which is considered one of the main marker compounds of treemoss. This is also corroborated by the detectable amounts of UA, which is not a major component of treemoss. (Joulain and Tabacchi, 2009b) The ratio of isopropyl/ethyl homologs was about 2:1 for any of the compounds. It is thus logical to hypothesize that a mixtures of alcohols may have been used during the solvent extraction phase to obtain the concrete. Poor chromatographic resolution was observed for the HPLC separation of ethyl haematommate/everninate homologs as well for the isopropyl derivatives.(Chittiboyina et al., 2020) Although HPLC-UV-MS would be desirable for a more comprehensive investigation of both volatile and non-volatile constituents, a better resolution of the commercial sample object of this study was achieved by GC–MS.

Overall, although an authentic reference material was not available, the chemical composition of the commercial sample analyzed was compatible with the one expected for untreated oakmoss absolute.

3.2. HTS-DCYA reactivity

Both the crude absolute and pure isolates along with readily available compounds were investigated using the *in chemico* HTS-DCYA method. This fluorescence-based assay enables the detection of thio-reactive compounds as corresponding fluorescence adducts either pure or in mixtures. (Avonto et al., 2016; Avonto et al., 2018) A concentration-dependent fluorescence response was observed for sample concentration between 1.25 and 5 mg/mL (Fig. 4). Four compounds were found to be reactive toward the model thiol DCYA, with IE > EE > ICH > IH. The remaining OMA constituents were found non-reactive in the HTS-DCYA. Aromatic aldehydes are known to be poor Michael acceptors in the presence of thiols, while they react preferentially with hard nucleophiles (*e.g.* amino groups) to form the corresponding Schiff's bases. The lack of reactivity in thio-trapping assay, especially in aqueous buffers, is therefore expected. The haematommates reactivity was in contrast with the lack of thio-reactivity of the atranols.

3.3. Direct peptide reactivity assay

The pure compounds were further investigated for peptide reactivity in the DPRA assay (Table 2). Because of limited sample availability, IE and ICH were not tested. MMP, ORC, MA, UA, and ATN were poorly or non-reactive toward the *Cys*-peptide. The most reactive compounds

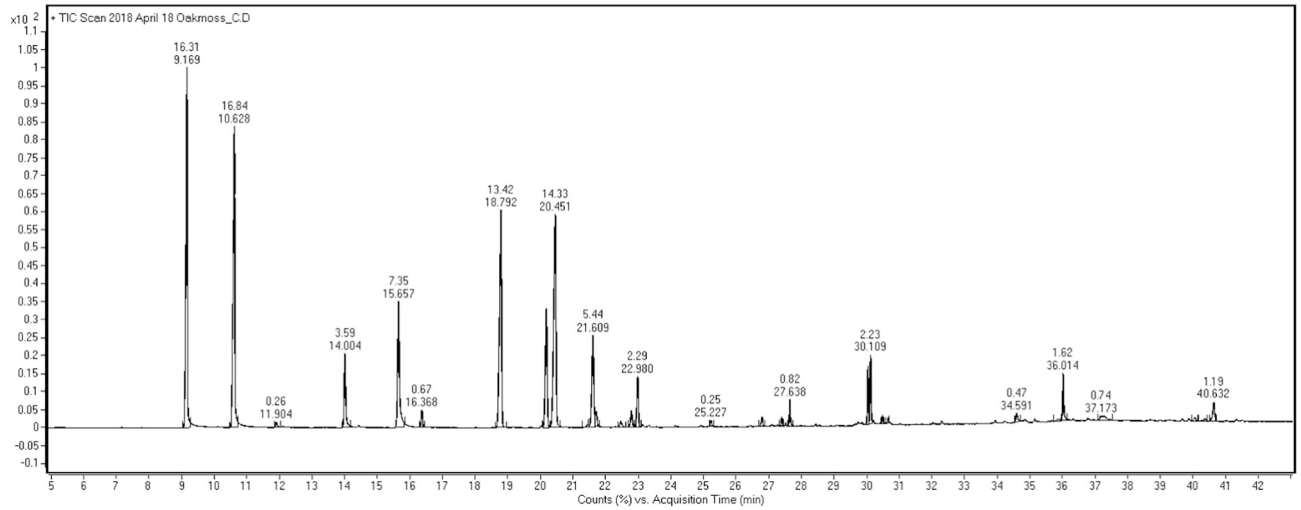

Fig. 1. GC–MS chromatogram of oakmoss sample OMA.

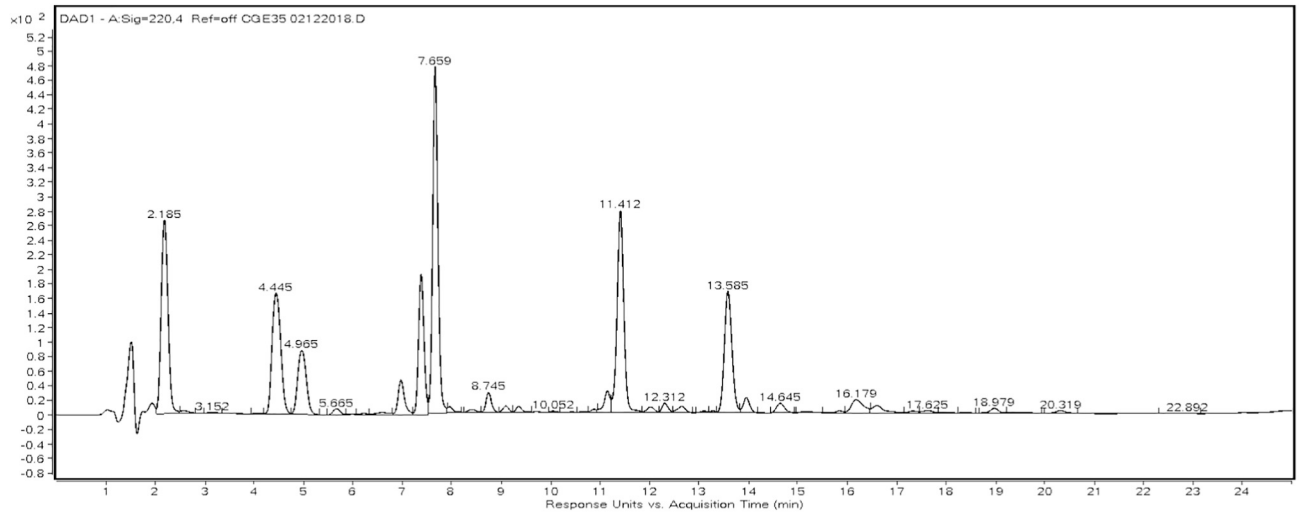

Fig. 2. HPLC-DAD-MS of oakmoss sample OMA.

Table 1
Identification of oakmoss main constituents and their relative distribution based on GC–MS analyses of the studied commercial product.

Peak	Compound	RT (min)	Area sum %	*m/z*
1	*3-Methoxy 5-methylphenol (MMP)*	9.169	16.31	138
2	*Orcinol (ORC)*	10.628	16.84	124
3	*Chloroatranol(ClATR)*	14.004	3.59	186
4	*Atranol (ATR)*	15.657	7.35	152
5	*Ethyl everninate (EE)*	18.792	13.42	210
6	*Isopropyl everninate(IE)*	20.180	6.66	224
7	*Methyl atratate(MA)*	20.451	14.33	196
8	*Ethyl orsellinate (EO)*	21.609	5.44	196
9	*Isopropyl haematommate (IH)*	22.980	2.29	238
10	*Isopropyl chloro-haematommate (ICH)*	27.397	0.29	272
11	*Usnic acid (UA)*	36.014	1.62	344

toward *Cys*-peptide were EE and IH, resulting in quantitative depletion, whereas moderate depletion was noticed in HTS-DCYA. The atranols and ethyl orsellinate were also reactive toward the *Cys*-peptide, while having only marginal reactivity in DCYA, and hence classified as positive. Echoing the dermato-toxicological outcome with oakmoss (Johansen et al., 2006), the halogenated ClATR resulted in 2.2 times higher depletion compared to the non-halogenated ATR.

In case of *Lys*-peptide depletion, the aldehydes and dimers resulted in stronger depletion. As anticipated, both atranols resulted in a stronger reactivity with *Lys*-peptide compared to *Cys*-peptide. In a similar fashion, usnic acid and atranorin were found reactive in *Lys*-DPRA whereas, non-reactive in *Cys*-DPRA. In general, the high-reactivity in *Lys*-DPRA does not necessarily reflect their reactivity, as chemical degradation in pH 10 buffer was observed within 24 h. ATR, ClATR, MA, and UA were especially unstable, resulting in the generation of degradation peaks within 24 h of reaction. It seems that compounds most reactive toward *Lys*-heptapeptide were also highly unstable. It is thus hard to distinguish between *Lys*-depletion driven by effective reactivity of the compounds *per se*, or by the generation of more reactive compounds upon incubation in the reaction buffer.

Based on averaged data, all the compounds with the exception of MMP and MA were classified as positive. Isopropyl haematommate was the most reactive compound, resulting in higher peptides depletion toward both *Cys*- and *Lys*- heptapeptides.

3.4. KeratinoSens™ and h-CLAT

According to the AOP for skin sensitization, the second key event is the elicitation of inflammatory response in the keratinocytes. The pro-inflammatory potential of OMA and its constituents was therefore tested in keratinocytes according to OECD guidelines (OECD, 2018a). Because of limited sample availability, IE and ICH were not tested further *in vitro*. As shown in Table 3, the crude OMA extract activated NRF-2 keap pathway with an EC1.5 of 2.68 μg/mL. Among the constituents, ClATR and UA did elicit pro-inflammatory responses *in vitro* with EC1.5 of 2.58 and 2.86 μM, respectively, while the remaining six constituents did not elicit pro-inflammatory responses up to the highest tested concentration of 2000 μM. The positive control CINN was effective with an EC1.5 of 31.17 μM. However, both constituents ClATR and UA were more potent than cinnamaldehyde..

Activation of stress response in dendritic cells is considered the third KE in the biological cascade leading to the clinical manifestation of skin sensitization. Among validated methods to study KE3, the h-CLAT has been included in OECD guidelines (OECD, 2018b). In this method, a human monocytic leukemia cell line (THP-1) is used in place of dendritic cells. The current approach to non-animal classification of skin sensitizers takes into account the weight of evidence of at least 2 positive methods for different KEs. Based on this approach, two congruent results would be sufficient for binary classification. Of all the oakmoss compounds object of this study, only four were unambiguously identified based on DPRA and KS agreement. Therefore, h-CLAT was performed on all main compounds obtained from OMA. The OMA extract did not cause an increase of 200% or 150% in RFI of CD54 or CD86, respectively, and therefore was considered negative for both CD54 and CD86 expression. However, five constituents namely ORC, ATR, MA, EO and UA were positive for CD54 expression (causing an increase of 200% in RFI) but negative for CD86 expression as shown in Table 3. The positive control DNCB increased the expression of both CD54 and CD86. Out of the five constituents which were positive for CD54 in h-CLAT, four were negative in KeratinoSens™ indicating that they did not elicit the proinflammatory response (KE2) but did prompt the activation of KE3. On the other hand UA was the only constituent that showed a positive response through both KE2 and KE3, while MMP and ATN were negative in both assays (Table 3). None of the compounds tested resulted in increase of CD86 expression. The final classification based on a “2 out of 3” approach is summarized in Table 4. Only one KE1 result was considered for the final classification. When conflicting DPRA and HTS-DCYA classification was found, DPRA data were used for the “2 out of 3” classification (OECD, 2017), except for the crude extract where the DPRA was not applicable.

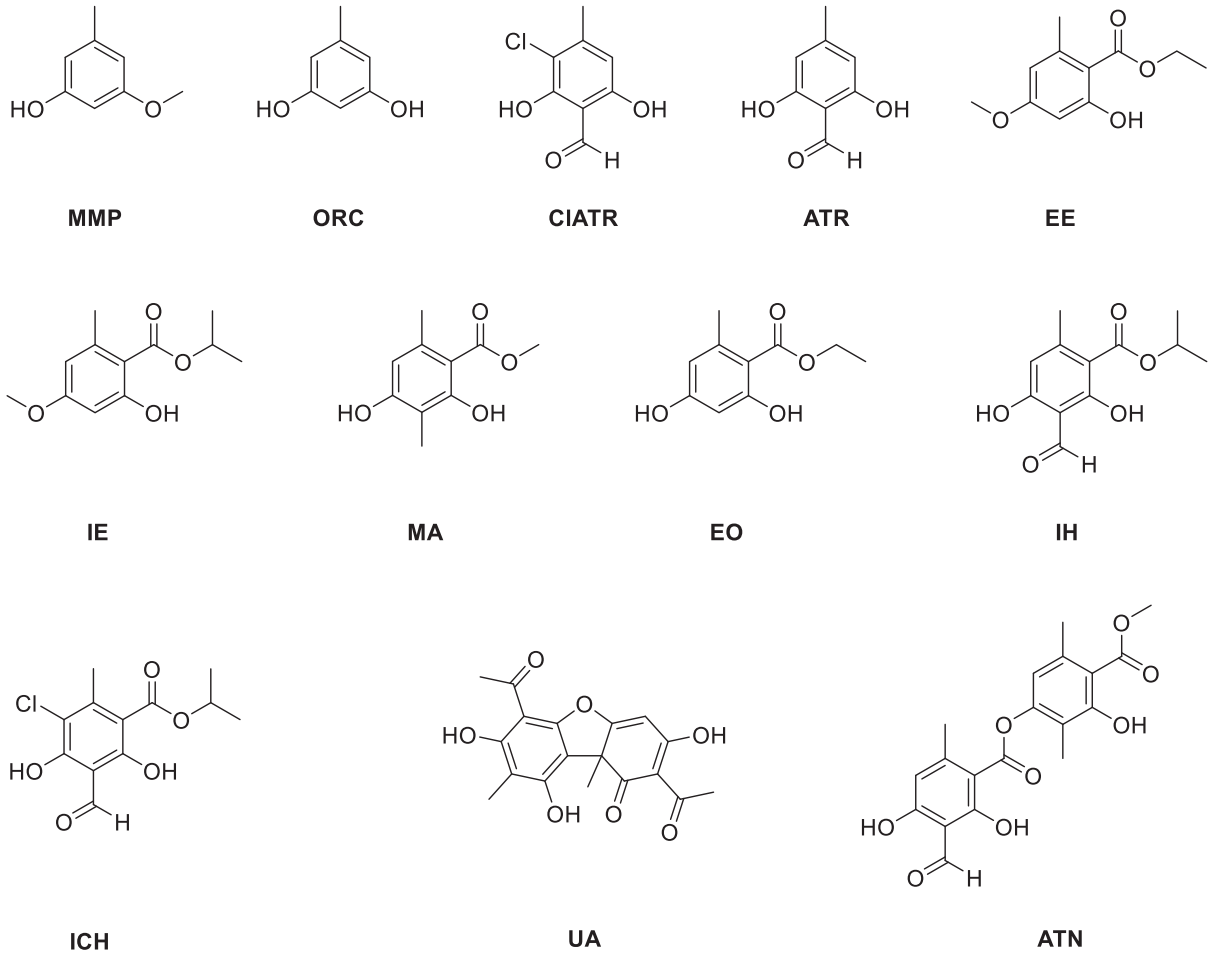

Fig. 3. Main constituents of commercial oakmoss absolute sample, OMA.

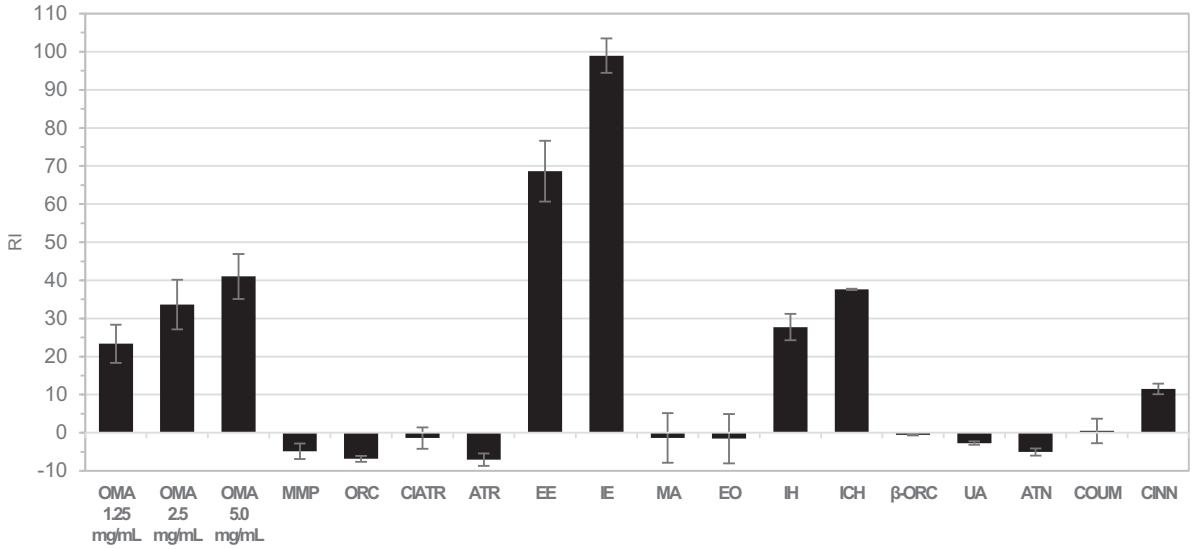

Fig. 4. HTS-DCYA results of CGE-35 and pure compounds. Coum = coumarin (negative control); cinn = cinnamaldehyde (positive control).

4. Conclusions

Investigation of skin sensitization potential of oakmoss absolute can be a challenge because of the difficulty in obtaining a standardized, authenticated OMA reference material, and the inconsistent distribution of marker compounds in commercial products. Lack of chemical consistency is a result of intrinsic chemical variability of the lichen, which is aggravated by potential contaminations occurring at harvesting and

Table 2
Direct peptide reactivity assay results of oakmoss constituents.

Compound	% depletion			Classification
	Cys-peptide	*Lys*-peptide	Average	
MMP	5.68 ± 1.87	2.53 ± 1.38	4.11	Negative
ORC	7.94* ±11.05	6.67 ± 1.77	7.31	Positive*
ClATR	66.11 ± 8.27	85.57 ± 5.54	75.84	Positive
ATR	29.91 ± 11.51	76.71 ± 7.67	53.31	Positive
EE	99.83 ± 0.19	13.66 ± 1.90	56.75	Positive
MA	6.08* ±9.53	n.q.	6.08	Negative
EO	27.88 ± 2.03	2.32 ± 0.88	15.10	Positive
IH	99.96 ± 0.05	84.35 ± 2.37	92.15	Positive
UA	4.59 ± 0.41	21.16 ± 6.47	12.88	Positive
ATN	1.33 ± 5.39	70.48 ± 9.12	35.90	Positive
CINN	81.6 ± 8.9	65.7 ± 10.3	73.65	Positive

* borderline quantification; n.q. = non quantifiable due to overlapping peaks. Cinnamaldehyde (CINN) was the positive control.

storing stages, along with changes in chemical composition occurring during the manufacturing process. In the present work, an investigation focused on individual pure components (which may commonly occur in oakmoss absolutes) was thus carried out. For a long time, ATR and ClATR have been considered the sole culprits responsible for the high number of skin sensitization cases reported in patients upon contact with oakmoss containing products. Animal methods have been extensively used to corroborate such findings. As the replacement of animal tests with non-animal methods is becoming a reality, an alternative approach using integrated non-animal methods has been adopted. The combination of chemical and *in vitro* methods enabled the identification of multiple compounds that can represent a safety concern.

Based on weight of evidence, both ATR and ClATR were confirmed potential skin sensitizers using a combination of three OECD non-animal methods (Table 4). Also, potential new candidate sensitizers in oakmoss were identified in usnic acid (positive for every KE tested), ethyl orsellinate and orcinol.

Chloroatranol was chemically more reactive than atranol in the DPRA, a finding which is in line with the observation of higher elicitation capacity of ClATR compared to ATR. The potency of ClATR *in vivo* has indeed been found to be 2.2 times higher than that of atranol. (Johansen et al., 2006) ClATR was able to elicit inflammatory events in keratinocytes but not to cause an increase of CDs expression in h-CLAT.

Overall, MMP and MA were the least reactive compounds, both *in chemico* and *in vitro*. Methyl atratate, also known as veramoss or evernyl, is considered one of the main characteristic fragrance components of the oakmoss absolute-containing products.

The results herein presented endorsed the finding that atranol and chloroatranol remain among the main compounds of concern in OMA ingredients, although not the only ones. Two minor compounds isolated from the commercial OMA were found to be potent sensitizers *in chemico* and therefore more investigations should be performed. The chemical reactivity of EE and IH was remarkable and overall stronger than both regulated atranols. If the chemical reactivity was to be supported by further evidence *in vitro*, these two compounds should also be regarded as a potential cause of adverse effects associated with OMA topical use.

Treatment of oakmoss absolute to reduce the atranols levels is plausibly lowering other potential allergenic constituents as well, but compounds such as EE and EO may still be retained in the sample after selective treatment. Multiple minor compounds have been found here to be both chemically reactive *and* capable of eliciting further inflammatory pathways in cellular models. It is also worth noting that some of the reactive compounds are not consistently present in common OMA, as they seem to be minor byproducts contingent on the manufacturing process. The evidence herein reported stress, one more time, how the manufacturing quality of botanical ingredients is important for safety as much as for quality and efficacy, and attention to the overall chemical fingerprinting should be addressed in OMA containing formulations.

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Table 4
Binary classification results and weight of evidence for OMA constituents.

Compound	HTS-DCYA*	DPRA	KS	h-CLAT	Classification
MMP	0	0	0	0	Negative
ORC	0	1	0	1	Positive
ClATR	0	1	1	0	Positive
ATR	0	1	0	1	Positive
EE	1	1	n.t.	n.t.	n.a.
MA	0	0	0	1	Negative
EO	0	1	0	1	Positive
IH	1	1	n.t.	n.t.	n.a.
UA	0	1	1	1	Positive
ATN	0	1	0	0	Negative
OMA	1	n.a.	1	0	Positive

1 = positive; 0 = negative; n.t. = not tested; n.a. = not applicable.
* Not considered for “2 out of 3” classification, except for OMA.

Table 3
KS and h-CLAT results of OMA and principal constituents.

Compound	KeratinoSens™		h-CLAT			
	EC 1.5 (μM)	Classification	CV75 (μg/mL)	CD54	CD86	Classification
OMA	2.68 ± 0.12	Positive	125	–	–	Negative
MMP	>2000	Negative	250	–	–	Negative
ORC	>2000	Negative	250	+	–	Positive
ClATR	2.58 ± 0.66	Positive	15.625	–	–	Negative
ATR	>2000	Negative	31.25	+	–	Positive
EE	n.t.	–	n.t.	–	–	–
MA	>2000	Negative	62.5	+	–	Positive
EO	>2000	Negative	62.5	+	–	Positive
IH	n.t.	–	n.t.	n.t.	n.t.	–
UA	2.86 ± 1.0	Positive	62.5	+	–	Positive
ATN	>2000	Negative	1000	–	–	Negative
*CINN**	31.17 ± 7.47	Positive	–	–	–	Positive
*DNCB**	–	–	4.0	+	+	Positive

* positive control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Andersen, F., Andersen, K.H., Bernois, A., Brault, C., Bruze, M., Eudes, H., Gadras, C., Signoret, A.C.J., Mose, K.F., Müller, B.P., 2015. Reduced content of chloroatranol and atranol in oak moss absolute significantly reduces the elicitation potential of this fragrance material. Contact Dermatitis 72, 75–83.

Anon, 2009. International Fragrance Association 43rd Amendment to the IFRA Code of Practice. Standard – Oakmoss Extracts.

Aptula, A., Patlewicz, G., Roberts, D., 2005. Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. Chem. Res. Toxicol. 18, 1420–1426.

Avonto, C., Chittiboyina, A.G., Rua, D., Khan, I.A., 2015. A fluorescence high throughput screening method for the detection of reactive electrophiles as potential skin sensitizers. Toxicol. Appl. Pharmacol. 289, 177–184.

Avonto, C., Chittiboyina, A.G., Wang, M., Vasquez, Y., Rua, D., Khan, I.A., 2016. *In chemico* evaluation of tea tree essential oils as skin sensitizers: impact of the chemical composition on aging and generation of reactive species. Chem. Res. Toxicol. 29, 1108–1117.

Avonto, C., Chittiboyina, A.G., Sadrieh, N., Vukmanovic, S., Khan, I.A., 2018. *In chemico* skin sensitization risk assessment of botanical ingredients. J. Appl. Toxicol. 38, 1047–1053.

Basketter, D.A., Sanders, D., Jowsey, I.R., 2007. The skin sensitization potential of resorcinol: experience with the local lymph node assay. Contact Dermatitis 56, 196–200.

Bernard, G., Giménez-Arnau, E., Rastogi, S.C., Heydorn, S., Johansen, J.D., Menné, T., Goossens, A., Andersen, K., Lepoittevin, J.-P., 2003. Contact allergy to oak moss: search for sensitizing molecules using combined bioassay-guided chemical fractionation, GC-MS, and structure-activity relationship analysis. Arch. Dermatol. Res. 295, 229–235.

Chittiboyina, A.G., Avonto, C., Rua, D., Khan, I.A., 2015. Alternative testing methods for skin sensitization: NMR spectroscopy for probing the reactivity and classification of potential skin sensitizers. Chem. Res. Toxicol. 28, 1704–1714.

Chittiboyina, A.G., Wang, M., Avonto, C., Parcher, J.F., Khan, I.A., 2020. Chromatographic analyses and unambiguous identification of atranol-like secondary metabolites in oakmoss absolute. Flavour Fragr. J. https://doi.org/10.1002/ffj.3578.

Dahlquist, I., Fregert, S., 1980. Contact allergy to atranorin in lichens and perfumes. Contact Dermatitis 6, 111–119.

ECVAM, 2014. EURL ECVAM Recommendation on the Keratinosens Assay for Skin Sensitization Testing. European Union Reference Laboratory for Alternatives to Animal Testing.

Ehret, C., Maupetit, P., Petrzilka, M., Klecak, G., 1992. Preparation of an oakmoss absolute with reduced allergenic potential. Int. J. Cosmet. Sci. 14, 121–130.

Gonçalo, S., Cabral, F., Gonçalo, M., 1988. Contact sensitivity to oak moss. Contact Dermatitis 19, 355–357.

Johansen, J.D., Bernard, G., Gimenez-Arnau, E., Lepoittevin, J.-P., Bruze, M., Andersen, K.E., 2006. Comparison of elicitation potential of chloroatranol and atranol - 2 allergens in oak moss absolute. Contact Dermatitis 54, 192–195.

Joulain, D., Tabacchi, R., 2009a. Lichen extracts as raw materials in perfumery. Part 1: oakmoss. Flavour Fragr. J. 24, 49–61.

Joulain, D., Tabacchi, R., 2009b. Lichen extracts as raw materials in perfumery. Part 2: treemoss. Flavour Fragr. J. 24, 105–116.

Kirk-Othmer, 2012. Kirk-Othmer Chemical Technology of Cosmetics. John Wiley & Sons.

Larsen, W.G., 1977. Perfume dermatitis. Arch. Dermatol. 113, 6.

Mowitz, M., Zimerson, E., Svedman, C., Bruze, M., 2013. Patch testing with serial dilutions and thin-layer chromatograms of oak moss absolutes containing high and low levels of atranol and chloroatranol. Contact Dermatitis 69, 342–349.

Mowitz, M., Svedman, C., Zimerson, E., Bruze, M., 2014. Usage tests of oak moss absolutes containing high and low levels of atranol and chloroatranol. Acta Derm. Venereol. 94, 398–402.

OECD, 2017. Guidance Document on the Reporting of Defined Approaches and Individual Information Sources to be Used within Integrated Approaches to Testing and Assessment (IATA) for Skin Sensitisation, OECD Series on Testing and Assessment, No. 256. OECD Publishing, Paris. https://doi.org/10.1787/9789264279285-en.

OECD, 2018a. Test No. In: 442D: *In vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, OECD Guidelines for the Testing of Chemicals, Section 4. OECD publishing, Paris.

OECD, 2018b. Test No. 442E: *In vitro* Skin Sensitisation: In Vitro Skin Sensitisation Assays Addressing the Key Event on Activation of Dendritic Cells on the Adverse Outcome Pathway for Skin Sensitisation, OECD Guidelines for the Testing of Chemicals, Section 4. OECD publishing, Paris.

OECD, 2020. Test No. In: 442C: *In chemico* Skin Sensitisation: Assays Addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins, OECD Guidelines for the Testing of Chemicals, Section 4. OECD publishing, Paris. https://doi.org/10.1787/9789264229709-en.

Rastogi, S.C., Bossi, R., Johansen, J.D., Menné, T., Bernard, G., Giménez-Arnau, E., Lepoittevin, J.P., 2004. Content of oak moss allergens atranol and chloroatranol in perfumes and similar products. Contact Dermatitis 50, 367–370.

Sandberg, M., Thune, P., 1984. The sensitizing capacity of atranorin. Contact Dermatitis 11, 168–173.

SCCP, 2008. Opinion on Oakmoss/Tree Moss (Sensitisation Only).

Schulz, H., Albroscheit, G., 1989. Characterization of oakmoss products used in perfumery by high-performance liquid chromatography. J. Chromatogr. A 466, 301–306.

Ter Heide, R., Provatoroff, N., Traas, P.C., De Valois, P.J., Van der Plasse, N., Wobben, H. J., Timmer, R., 1975. Qualitative analysis of the odoriferous fraction of oakmoss (*Evernia prunastri*). J. Agric. Food Chem. 23, 950–957.

Thune, P., Solberg, Y., McFadden, N., Stærfeet, F., Sandberg, M., 1982. Perfume allergy due to oak moss and other lichens. Contact Dermatitis 8, 396–400.

Vinken, M., 2013. The adverse outcome pathway concept: a pragmatic tool in toxicology. Toxicology 312, 158–165.