Chemical compounds responsible for skin allergy to complex mixtures: how to identify them?

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Abstract

In the cosmetics industry, various natural complex mixtures such as botanical extracts or essential oils are used. In addition to that, finished consumer products may contain a number of constituents of natural origin but many products derived from organic synthesis too. Hence, finding skin sensitizers within this myriad of chemicals is an arduous task. Nowadays, methods validated by European dedicated instances to evaluate the allergenicity of chemicals are incapable to predict the sensitization potential of complex mixtures, even if research has progressed a lot in this direction recently. Accordingly and in this context, to identify precisely the culprit(s) responsible for skin sensitization to the mixtures is essential for risk assessment. This review is a short summary of approaches that allow identifying allergens in chemical mixtures such as bioassay-guided chemical fractionation, structure-activity relationships studies and recent methods allowing identification of reactive intermediates in natural extracts exposed to air oxidation. It is shown that a big progress has been accomplished, even if the identification of sensitizers in complex mixtures continues to be puzzling.

Keywords

Skin sensitizers, hazard identification, mixtures, bioassay-guided fractionation, structure-activity relationships, chemical reactivity.

1. Introduction

Allergic contact dermatitis (ACD) is an everyday occupational and environmental issue. It is a delayed hypersensitivity reaction produced by an extensive range of reactive chemicals or allergens, natural or synthetic man-made, after recurrent contact with the skin. It is the most frequent expression of immunotoxicity in humans. Prevalence is increasing worldwide, being a flagrant menace to public health. Social and regulatory pressures have strengthened with European Union legislation, in a way that exposure to chemicals and the risk of skin sensitization is today an indispensable regulatory issue for the industry. It has become thus central to predict the sensitization potential of chemicals before their use in consumer products to perform risk assessment.

Assessment of the skin sensitization potential of chemicals has improved very much thanks to the development of non-animal assays owing to regulation necessities and ethical problems [1, 2]. These methods have been developed focusing on the first three main events of the adverse outcome pathway (AOP) defined for the sensitization phase, protein binding, keratinocytes and dendritic cells activation [3]. Among others, the direct peptide reactivity assay (DPRA) addressing protein binding, the KeratinoSens^{TM} keratinocytes activation and the human Cell Line Activation Test (h-CLAT) concentrating on dendritic cell activation have been approved by the Organization for Economic Co-operation and Development (OECD) for testing health effects of chemicals [4-6]. Though, these assays were developed basically for the test of pure substances. The fragrance and cosmetics industry uses naturally derived complex mixtures such as essential oils and botanical extracts, some of them reported as responsible of skin allergy [7]. REACH and EU regulations require not only pure substances to be evaluated and registered but also mixtures extracted form natural sources. Moreover, since most perfumes contain mixtures of fragrance ingredients, many of them having a skin-sensitizing capacity, consumers are exposed to mixtures of allergens when they use products that contain perfume.

Thus, a big deal now is finding methods able to evaluate the sensitizing potential of mixtures. This is a problematic task in existing methods. The *in vivo* local lymph node assay (LLNA), assessing sensitization to chemicals in mice, has been used for assessment of some essential oils. It was concluded that, generally, the potency of the essential oil does not differ particularly from that of its main components [8]. The DPRA evaluated at first glance chemical reactivity of mixtures in a very preliminary approach. Combinations of fragrance aldehydes hydroxycitronellal-citral and citral-cinnamaldehyde were studied. The reactivity towards DPRA peptides was compared with that of the single constituents. The chemical described as the most potent sensitizer was leading the reactivity in the mixtures. Consequently, it was hypothesized that DPRA would estimate the reactivity of the mixture similar to that of the stronger sensitizer component [9]. KeratinoSens^{TM} was attempted as an *in*

vitro assay for plant extracts [10]. As proof of concept for testing botanical extracts, they were artificially spiked with citral, cinnamaldehyde and isoeugenol at different doses. The extracts were negative in the assay but positive when spiked with the sensitizers. Recently, the combination KeratinoSens™ with h-CLAT has been studied by comparing the detection limits in both assays of 146 skin sensitizers present in very low concentrations in botanical extracts with LLNA values of EC_3 [11]. More innovative is the study performed to identify skin sensitizers in henna products using the GARD™ skin assay. Genomic Allergen Rapid Detection (GARD) platform is a cell-based assay that uses the innate recognition of xenobiotics by dendritic cells, measured by monitoring genomic biomarkers [12]. In the context of henna-based hair dye products, GARD™ skin has been further combined with validated tests such as the micro-direct peptide reactivity assay, the HaCaT keratinocytes-associated IL-18 assay and the U937 cell line activation test (USENS) [13]. In general, assessed end-points increased in henna products when compared to hair dye constituents tested alone. Also, we reported that existence of fragrance compounds together in a consumer product leads to increased sensitization potency (cocktail effect due to the mixture). We studied the influence when mixing fragrance allergens on the sensitization process to the individual compounds finding that allergen mixtures enhance both induction and elicitation of ACD [14]. We carried out similar studies with atranol and chloroatranol, key allergens of oak moss absolute, showing that the mixture in the natural extract was more potent at both sensitization and challenge than atranol and chloroatranol alone [15]. Finally, the ability of the SENS-IS assay, which uses an EpiSkin™ large model of 3D reconstructed human epidermis, to detect such mixture effects was evaluated very recently showing that it is efficient for detecting skin sensitizing danger in complex mixtures and final products [16].

Efforts continue to be done. However, each of the above methods is still unable to fully predict the skin sensitization potential of mixtures. Thus, hazard identification continues to be crucial for risk assessment purposes. An essential step to prevent ACD is to identify the nature of chemicals responsible for skin allergy in the consumer, to build initiatives regulating exposure in a way that skin sensitization does not occur. This manuscript reviews methodologies to identify skin sensitizers in mixtures, such as bioassay-guided chemical fractionation, structure-activity relationships studies (SARs) and methods based on chemical reactivity identifying allergens in natural extracts exposed to air oxidation.

2. Bioassay-Guided Fractionation (BGF)-Combination with SARs

BGF is usual to identify bioactive compounds [17]. It is based on the isolation of a chemical by a step-by-step extraction of the natural extract components upon physicochemical properties and on the

evaluation of the biological activity, followed by rounds of separation and testing. BFG is applied when a given crude prepared from a natural material is pondered to be "active" in a specific *in vitro* assay. The aim is to identify the culprits for the *in vitro* activity. After fractionating the crude extract by chromatographic techniques (i.e. column chromatography, high-performance liquid chromatography) fractions are evaluated in the *in vitro* assay. These steps are reiterated with active fractions until pure active compounds are obtained.

BGF has also been applied to perfume mixtures giving ACD symptoms to find the responsible of skin sensitivity declared by the patient to that perfume. Ethanol of the commercial product is evaporated and the concentrate is chemically fractionated. Fractions are evaluated on the patient by patch testing and/or by repeated open application test (ROAT). Fractions giving a positive reaction are re-fractionated, and the new ones obtained are evaluated again on the patient. This is reiterated until a positive fraction containing one or two single compounds easy to identify by spectroscopic techniques is obtained (Figure 1a). In a clinical case where a 44-year-old woman developed ACD to her eau de toilette we identified the offending allergen by BGF [18]. She presented at the dermatological clinic with axillary rash after using a perfumed deodorant. Using the eau de toilette of the same brand she developed neck and trunk rash. After patch testing, she was positive to the deodorant and the eau de toilette, but negative to fragrance mix I (FMI), the diagnostic tool dermatologists employ together with fragrance mix II (FMII) to elucidate fragrance allergy cases.

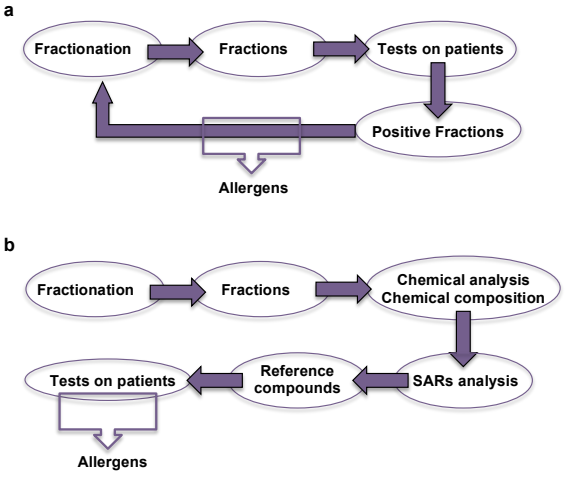


Figure 1. Identification of allergens in mixtures. (**a**) BGF. (**b**) BFG and structure-activity relationship analysis (SARs).

The concentrate of the eau de toilette obtained after evaporation was fractionated by column chromatography on silica gel giving three fractions tested on the patient in a ROAT. One fraction was

positive and was additionally chemically fractionated to give four sub-fractions tested on the patient. One sub-fraction was positive. It contained coumarin and ethyl vanillin. The patient was negative on testing to ethyl vanillin but positive to coumarin after two days of application. Coumarin was thus confirmed as the offending allergen.

The case of essential oils is much more complicated as they have multitude of constituents. Analytical surveys can be carried out. This is especially convenient when patients also react to individual chemicals. Analytical surveys can display the presence of such chemicals in the oils. Gas chromatography-mass spectrometry (GC-MS) is the most useful analytical tool as essential oils are mixtures of volatile odorous compounds. A very complete investigation by GC-MS has been conducted on essential oils used by aroma therapists with ACD [19]. For a patient sensitized from topical application of tea tree oil for therapeutic reasons [20], GC-MS analysis identified the presence of 1,8-cineole, and the patient was positive to this compound.

However, things are not that easy. The need to complement BGF with SARs became quickly evident in ACD to fragrances owing to the difficulty to obtain refined fractions. SARs fundamentals are that the mode of interaction of a chemical with a defined system is defined by the molecular structure. The biological activity of a compound is thus function of its structural-physicochemical properties.

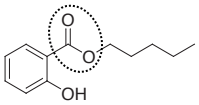
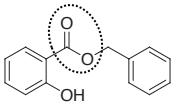
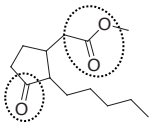
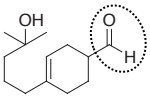
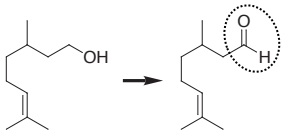
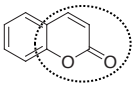
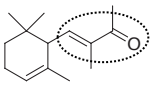
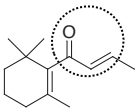
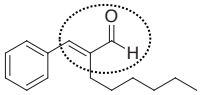
The first step in skin sensitization to a chemical is the formation of a covalent bond between the allergen after penetrating the epidermis and skin proteins to form an immunogenic complex. This occurs classically through nucleophile-electrophile mechanisms. Many allergens contain in the chemical structure an electrophilic functional group reacting with nucleophilic amino acids [21]. It can also happen in some cases through radical mechanisms [22]. Therefore, if a chemical is able to react with a protein, then it has the potential to be a contact allergen. Today there is a considerable knowledge on the chemical functions related to skin sensitization. This knowledge has been the basis for the establishment of chemical rules, the so-called “structural alerts”. A “structural alert” is a total or partial chemical structure known to present a risk. The presence of a “structural alert” in a molecule is a sign of a faculty to modify skin proteins and thus act as a skin sensitizer. In our laboratory we have developed an approach to identify fragrance sensitizers present in mixtures based on the combination of BGF, chemical analysis and SARs. In a few words, the mixture is chemically fractionated and the resulting fractions are tested on the patient(s) allergic to the mixture. The composition of positive fractions is analyzed (GC-MS, LC-MS). From the constituents detected, a SARs analysis selects molecules with a suspected sensitizing potential due to the presence of structural alerts. These molecules are then directly tested on the patient(s) for the identification of the sensitizer(s) (Figure 1b).

This approach avoids iterative fractionation/patient testing sessions that are time consuming and require considerable effort by the patients.

An eau de toilette

BGF, chemical analysis and SARs were applied to identify in an eau de toilette fragrance allergens not included in FMI/FMII [23]. The clinical case was a 50-year-old woman that developed severe eczema during the summer 5 months earlier. She presented with dermatitis over the neck, upper thorax and retro-auricular area. The dermatitis worsened after sun exposure, the patient complaining of severe itching. With the suspicion of cosmetic ACD patch tests were performed with the European standard series and with own cosmetic products. The standard series (including FMI) was negative. A strong positive reaction was elicited by her eau de toilette. Column chromatography on silica gel of the concentrate obtained from evaporating the eau de toilette gave 5 fractions (F1-F5) used for testing the patient at a concentration equivalent to the final formulation. The physical appearance of the concentrate presented also colour components that could not be isolated and had been added to the final formulation. The producer company confirmed this and sent us FD&C Yellow no. 5, FD&C Red no. 4, and the photo-screen agent Uvinul D-50®. No reactions to FD&C Yellow no. 5 and FD&C Red no. 4 were observed. The patient had a strong reaction to Uvinul D-50®. Also, F4 gave a severe reaction followed by F1. The chemical composition of F1-F5 was analysed by GC-MS. Despite the fact that not all GC-peaks could be assigned to a specific compound (absence of reference mass-spectra in the MS-library; mixed GC peak), we identified 25 compounds for which the sensitizing potential was evaluated by the presence in the chemical structure of structural alerts [24]. This way, 9 compounds contained a structural alert with potential ability to react with skin proteins and thus behave as a skin sensitizer and were patch tested on the patient (Table 1). The patient was positive to hydroxyisohexyl-3-cyclohexene carboxaldehyde (Lyral®), α-hexyl cinnamaldehyde and α-damascone. GC chromatograms of the eau de toilette, F1 and F4 are shown in Figure 2. F4 contained exclusively hydroxyisohexyl-3-cyclohexene carboxaldehyde and 2,2',4,4'-tetrahydroxybenzophenone (benzophenone-2, Uvinul D-50®). F1 contained α-hexyl cinnamaldehyde and α-damascone. This way, culprits for ACD in this patient were elucidated.

Table 1. The 9 compounds with a structural alert and patch test results

Compound	Chemical structure and structural alert[a]		Conc.[b]	D2	D4	D7
pentyl salicylate		ester	2%[c]	–	–	–
benzyl salicylate		ester	1%[c]	–	–	–
methyldihydrojasmonate		ester and ketone	2%	–	–	–
Lyral®[d]		aldehyde	2%[c]	+++	+++	++
citronellol		alcohol susceptible to metabolisation into an aldehyde	1%[c]	–	–	–
coumarin		α,β-unsaturated lactone	5%[c]	–	–	–
α-isomethylionone		α,β-unsaturated ketone	2%	–	–	–
α-damascone		α,β-unsaturated ketone	2%	+++	+++	++
α-hexyl cinnamic aldehyde		α,β-unsaturated aldehyde	2%[c]	++	+++	+

[a] The electrophilic chemical group in the molecule with ability to form covalent conjugates with skin proteins is distinguished with a dotted circle line.
[b] Patch test concentration in petrolatum.
[c] According to de Groot [25].
[d] The commercial product is a mixture of 2 isomers. The chemical structure of the major isomer, the 4-(4-hydroxy-4-methyl-pentyl)-3-cyclohexene carboxaldehyde (70%), is shown.

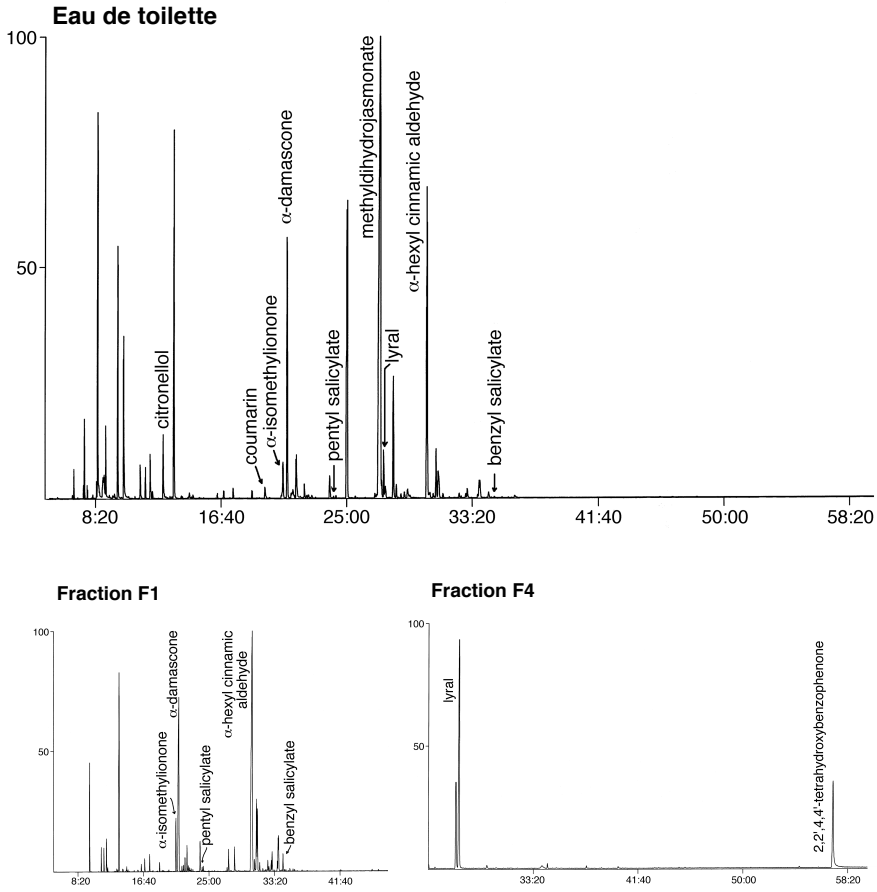


Figure 2. GC chromatograms of the eau de toilette and fractions F1 and F4.

Oak moss natural extract

Oak moss absolute, from the lichen *Evernia prunastri* (L.) Arch, has been used broadly in perfumery because of its woody aroma and fixative properties. It is the most common allergen among the constituents of FMI [26]. It is obtained by extraction of the harvested lichen with hydrocarbon solvents followed by treating the concrete with a mixture of alcohols. In the 70's-80's the chemical composition was analyzed and the allergenicity of the major components evaluated [27, 28]. Sensitivity to oak moss was related to phenylbenzoates such as atranorin and evernic acid, but also usnic acid. For long time benzene was used to prepare the concrete, but more polar solvents changing the composition of the absolute replaced it. Constituents of oak moss absolute obtained from the new extraction procedures and related allergenicity were then not clear. We applied BGF and SARs to these new extracts [29]. Patients with known ACD to oak moss were recruited for the study. After a first fractionation of the extract and testing, attention was focused on the strongest eliciting fraction. GC-MS chemical analysis identified 8 compounds derived from a resorcinol structure. SARs suggested that all

needed to be considered potential sensitizers. To avoid too many tests on the patients, we selected for testing the most representative molecules covering the different molecular structures. Following this procedure, atranol and chloroatranol were identified as the major eliciting chemicals, and methyl-β-orcinol carboxylate as a minor one. Atranol and chloroatranol are degradation products of atranorin and chloroatranorin during oak moss processing (Figure 3). Methyl-β-orcinol carboxylate, also obtained during this processing, is responsible for the characteristic earthy-moss-like odor.

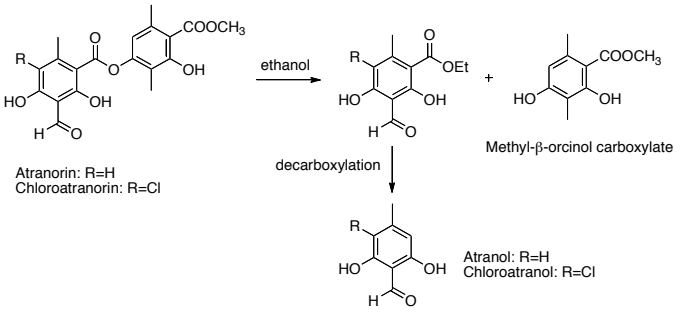


Figure 3. Atranol and chloroatranol from atranorin and chloroatranorin during oak moss processing.

Later on, we proved by exposure assessment and dose-response elicitation studies that atranol and chloroatranol elicit reactions at very low levels of exposure [30-32]. As a consequence, the European Union has issued regulation 2017/1410 banning the use of atranol and chloroatranol in consumer products [33]. From August 2019 cosmetic products containing these substances shall not be placed on the Union market, and from August 2021 shall not be made available on the Union market.

3. Complex mixtures and chemical reactivity

During the last decade, a step forward has been made in the identification of sensitizers in complex mixtures based on their chemical reactivity. Approaches based on reactivity with cysteamine derivatives as model of nucleophiles in the skin have been developed. The NMR-dansyl cysteamine (NMR-DCYA) and the high throughput screening-DCYA (HTS-DCYA) methods evaluate the ability of chemicals to covalently bind to DCYA. To study the ability of the test molecule to initiate haptenation, the model thiol DCYA operates as surrogate of nucleophilic residues in skin proteins. DCYA allows thus reactivity of electrophilic and other thio-reacting species. In the NMR-DCYA assay, electrophilic depletion over time is quantified by proton NMR [34]. Quantification is carried out based on the depletion of the test molecule in contrast with present validated methods that are based on the depletion of the nucleophile species (i. e. DPRA). The HTS-DCYA method permits a sensitive detection of electrophilic compounds in a high throughput screening using fluorescence assays [35]. The test molecule is incubated with DCYA and activated to initiate a covalent binding. The

fluorescence response of DCYA-adducts is quantified. The amount of reacting species is described as a reactive index (RI), correlating with the amount of DCYA-adducts found. A drawback of these methods is consideration of reactive electrophilic sensitizers exclusively, without considering sensitizers able to modify skin proteins through other kind of mechanisms such as the intervention of radical intermediates [22].

Aged tea tree oil

Tea tree oil (TTO) is obtained from leaves and terminal branchlets of *Melaleuca alternifolia*, *Melaleuca linariifolia* or *Melaleuca dissitiflora* by steam-distillation [36]. It is a colorless to yellow liquid with a coniferous minty-camphor odor. It has been reported as anti-inflammatory, analgesic, antimicrobial, biocidal and antitumoral. Yet, TTO causes ACD, the first case reports being published in the 90s from Australia where the oil is produced.

TTO is catalogued as a moderate sensitizer in the LLNA and main constituents lack "structural alerts" to act as skin sensitizers [37-39]. The fact is that TTO in contact with air suffers photo-oxidation aging processes. Air exposure results in a 3-fold increase in the sensitization potency. Degradation products that are strong sensitizers are this way obtained. Most important sensitizers in aged TTO appear to be terpinolene, ascaridole, α-terpinene and its oxidation products, 1,2,4-trihydroxymenthane, α-phellandrene, *d*-limonene and myrcene.

In a recent study, aged TTOs were used to evaluate the applicability of the HTS-DCYA assay [40]. The global approach of the methodology is shown in Figure 4. Chemical composition of the oils after aging was analyzed by GC-MS. An increase in the amount of *p*-cymene was noticed and considered as an indicative marker for aging, together with the formation of several peroxide derivatives. For "authentic" TTOs higher reactivity with *p*-cymene was observed, even if *p*-cymene was found to be non-reactive in the HTS-DCYA assay. TTOs with high peroxide content resulted in highest RI values. The oxidized sample giving the higher RI value was analyzed to get more information on the reaction products, and thus the reactive species and potential sensitizers. Major DCYA adducts were then isolated and chemical structures elucidated by NMR and/or MS. The major peak matched with a mixture of diastereomers (*m/z* 436) obtained by 1,4-Michael addition of DCYA on 4-hydroxy-4-methylcyclohex-2-en-1-one (Figure 5). This α,β-unsaturated ketone (*m/z* 126) is a well known degradation product of ascaridole (Asc), endoperoxide derived from α-terpinene autoxidation.

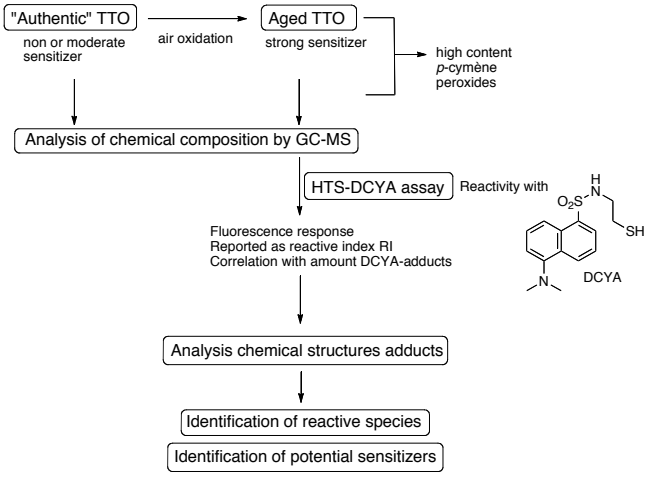


Figure 4. Global approach of the HTS-DCYA assay: example of aged TTO.

Cleavage of the O-O bond of Asc produces the loss of isopropyl radicals (iPr$^{\bullet}$) forming this way the reactive ketone. Indeed, recently we have demonstrated that Asc is activated in the skin to form alkoxyl radicals that further rearrange to carbon radicals and reactive electrophilic species, and that under these conditions dendritic cell activation is promoted [41]. Peaks with *m/z* 478 were also observed. Based on the mass fragmentation pattern, adducts could originate from isoascaridole *via* the nucleophilic thiol ring opening of the oxirane moiety. Isoascaridole is also known as a by-product obtained after decomposition of Asc [42]. α-Terpinene is thus probably one critical component of TTO that undergo singlet oxygen addition to give Asc that can further form electrophilic species such as the α,β-unsaturated ketone or isoascaridole. Such intermediates could be responsible for the concomitant irritancy and co-sensitization observed with increased concentrations of Asc and Asc-containing essential oils [43].

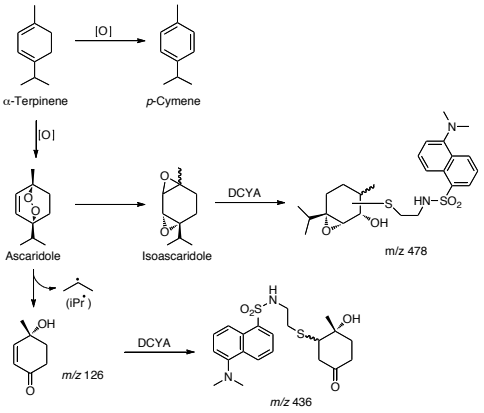


Figure 5. Suggested pathway for the formation of DCYA adducts in aged TTO.

This study demonstrates that formation of electrophilic species via radical degradation is one possible pathway that may explain reactivity and sensitization to aged TTOs. Assuming that many skin sensitizers are electrophilic compounds reacting with nucleophilic amino acids in the skin, this study represents as proof-of-concept how the HTS-DCYA assay may be used to analyze mixtures for the presence of reactive electrophilic compounds.

To end with this section it is worthy to mention that, even more advanced in this type of methodologies based on chemical reactivity, in a recent study NMR-DCYA and HTS-DCYA have been combined with BGF using the KeratinoSens^{TM} assay to elucidate the culprit of the sensitizing potential of German chamomile extract [44].

4. Conclusion

Hazard identification is the first step to prevent skin sensitization and ACD. It is thus compulsory to have reliable methods for the identification of chemicals with the potential to cause skin sensitization, ideally before they are put into the market but also present in consumer products. Many ingredients present in consumer products are complex mixtures such as essential oils or botanical extracts that may have sensitizing properties. Today, identification of culprits of the sensitizing potential of complex mixtures continues to be puzzling. This manuscript is a brief outline of methods existing to identify skin sensitizers in mixtures. BGF has been an established method for many years. Today, combination of BGF, clinical testing of patients with fractions, chemical composition analysis and SARs, is a valuable tool to identify allergens in fragrance mixtures. This last decade several researchers are bringing new fresh air with methods based on chemical reactivity and fluorescence, basically studying natural extracts after aging upon air oxidation. Still, evaluating the sensitizing potential of mixtures and identifying the culprits is far from being unravelled. Plus, following hazard identification it is critical to conduct skin sensitization risk assessment based on ingredient exposure, allergenic potency and dose-response studies. Even if successful efforts have been done, more are necessary to launch a strategy that lets assessing mixtures extracted from natural sources and active sensitizing components.

Abbreviations

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; Asc, ascaridole; BGF, bioassay-guided fractionation; DCYA, dansyl cysteamine; DPRA, direct peptide reactivity assay; FMI, fragrance mix I; FMII, fragrance mix II; GARD, genomic allergen rapid detection assay; GC-MS, gas chromatography-mass spectrometry; h-CLAT, human cell line activation test; LLNA, local lymph node assay; MS, mass spectrometry; NMR, nuclear magnetic resonance; OECD, Organization for Economic Co-operation and Development; RI, reactive index; ROAT, repeated open application test; SARs, structure-activity relationships; TTO, tea tree oil.

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