Identification of Allergens in Complex Mixtures and Products

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Abstract

Identifying contact allergens among the myriad of chemicals used in commercial products is an extremely arduous task. This is even more difficult in the case of components of complex mixtures such as natural extracts and essential oils. Today, non-animal alternative methods validated to assess the sensitizing potential of chemicals are, overall, unable to completely predict the skin sensitization potential of these complex mixtures of chemicals. Moreover, toxicological profiles are very difficult to extrapolate from the mere sum of the profiles of the individual constituents as required today. In

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Keywords

Bioassay-guided fractionation · Structure-activity relationships · In chemico methods · Fluorescence trapping · Chemical reactivity · Oak moss absolute · Tea tree oil

1 Introduction

Preventive measures are mandatory in the management of allergic contact dermatitis to a chemical. The first essential preventive step is hazard identification, in other words, to fully identify the nature of chemicals responsible for causing contact allergy in the consumer. It is then possible to create initiatives that regulate exposure to that chemical so that induction of skin sensitization does not take place and to improve risk assessment procedures. This confirms the necessity to have suitable, sensitive, and reliable methods for the prospective identification of novel chemicals that possess the potential to cause sensitization of the skin.

Identifying contact allergens among the myriad of chemicals used in commercial products can be an extremely arduous task. For instance, approximately 3000 fragrance ingredients, of synthetic or natural origin, are currently available to the perfumer for compounding a fragrance formula [1]. Thus, a single perfume or fragrance composition may consist of 10–300 different ingredients. And among them are natural extracts and essential oils. Essential oils are complex volatile odorous mixtures of terpenes, aldehydes, alcohols, esters, and ketones, widely used in aromatherapy (massaging, adding to bath, inhalers, etc.) and in the perfume and cosmetic industry. Because of their constant use, contact allergy has been increasingly recognized [2].

Assessment of the skin sensitization potential of chemical compounds, another essential toxicological end-point in safety evaluation measures, has made a great deal of progress during the last decade due to the encouragement of regulatory agencies to develop alternative methods to eliminate the use of animals [3, 4]. Several *in silico*, *in vitro*, and *in chemico* alternative methods have been developed to assess skin sensitization to chemicals following the ban on animal testing introduced by cosmetic legislation in Europe in 2013. Some of these methods, such as the *in chemico* direct peptide reactivity assay (DPRA), the *in vitro* ARE-Nrf2 luciferase assay (KeratinoSens™), and the human cell line activation test (h-CLAT), have been recently adopted by the Organization for Economic Co-operation and Development (OECD) for testing health effects of chemicals [5–7]. These tests were developed and validated for the testing of pure substances. However, REACH regulation requires not only pure substances to be registered but also registration of mixtures extracted from natural sources. Though, there is very little experience with testing of mixtures, and assessment of test material with more than one component has been proved to be an elusive task in existing methods. The *in vivo* local lymph node assay (LLNA) was used nevertheless to test a number of essential oils and authors stated that in general the potency of each essential oil did not differ significantly from that of its main individual components [8]. Also, the applicability of the KeratinoSens™ assay has been evaluated as a proof of concept for four plant extracts used in cosmetics, spiked with different doses of citral, cinnamaldehyde, and isoeugenol [9]. The extracts were negative in the test, whereas they were positive when spiked with the sensitizers. Finally, hapten-peptide reactivity-based DPRA was evaluated in a very preliminary approach to evaluate at a first sight chemical reactivity of mixtures. Two binary combinations of fragrance aldehydes were studied, hydroxycitronellal-citral/citral-cinnamaldehyde. Their reactivity towards DPRA peptides was compared with that of the single constituents. In general, the chemical described as the most potent sensitizer was driving the reactivity in the mixtures. Therefore, it could be hypothesized

that the DPRA would estimate the mixture chemical reactivity similar to that of the stronger sensitizer component [10]. Though, even if efforts continue to be done, each of the above non-animal alternative methods are, overall, still unable to completely predict the skin sensitization potential of complex mixtures of chemicals. In this context, hazard identification remains still one of the best solutions for risk assessment.

The present chapter describes different methodologies that exist today for the identification of contact allergens in complex mixtures and products, such as the use of bioassay-guided chemical fractionation, in combination with structure-activity relationships studies (SARs) and new techniques developed to trap and identify reactive intermediates in natural extracts during air oxidation. Practical applications such as eau de toilette cases, the allergenic natural extract oak moss, and sensitizing air oxidized tea tree oil are exposed.

Bioassay-Guided Fractionation

Bioassay-guided fractionation is a common used strategy for the identification of bioactive compounds and new drugs [11, 12]. It is based on the isolation of a pure chemical agent of natural origin by a step-by-step separation of extracted components upon differences in their physicochemical properties and assessing the biological activity, followed by a next round of separation and assaying. Typically, the process is initiated after a given crude, normally prepared by solvent extraction of the natural material, is considered to be "active" in a specific in vitro assay. The end-goal is the identification of compounds responsible for the observed in vitro activity. The way to that end is rather simple. After fractionating the crude extract, normally by chromatographic techniques such as column chromatography and high-performance liquid chromatography, fractions generated are tested in the in vitro assay. These steps are then repeated with active fractions until pure, active compounds are obtained for which the structure can be determined by using spectroscopic methods

such as nuclear magnetic resonance (NMR) and mass spectrometry (MS).

The same kind of methodology can be applied to allergenic perfume mixtures in order to find the compound(s) responsible for skin sensitivity declared by the patient(s) to that mixture. Ideally, the perfume mixture is chemically fractionated, and fractions obtained are tested on the patient(s) by patch testing and/or by repeated open application test (ROAT). Fractions giving a positive reaction are refractionated, and the new fractions obtained are tested on the patient(s). These steps are repeated until a positive fraction that contains one or two single compounds easily identifiable by spectroscopic techniques is obtained (Fig. 1a). An example can be given by a study in which the offending allergen in a patient sensitive to an eau de toilette but negative to the fragrance mix I (FMI) was identified [13]. A 44-year-old woman without previous skin problems developed an axillary rash using a perfumed deodorant. At the same time, she started to use the eau de toilette of the same brand and developed a rash on the neck and trunk. She was patch tested positive to the deodorant and the eau de toilette but was negative to FMI. The eau de toilette perfume concentrate was chemically fractionated by column chromatography on silica gel giving three fractions that were tested on the patient in a ROAT using ethanol as vehicle. The test material concentration was calculated so that it corresponded to the amount of the fraction present in the final formulation. Only one fraction gave a positive reaction and was further chemically fractionated to afford four subfractions that were tested on the patient. One subfraction confirmed a positive reaction. It was analyzed and found to contain simply coumarin and ethyl vanillin. The patient was negative on use testing to 1% ethyl vanillin in ethanol but was positive to 1% coumarin in ethanol after 2 days of application. This simple case of bioassay-guided fractionation on a fragrance mixture confirmed coumarin as the offending allergen. Today, coumarin, a widely used fragrance compound, is one of the ingredients of FMII introduced last decade as an additional screening tool to FMI [14, 15].

In the case of essential oils, analytical investigations on the components that are at the origin of

Fig. 1 Identification of allergens in complex mixtures. (a) Bioassay-guided fractionation. The mixture is chemically fractionated, and fractions obtained are tested on the patient(s) by patch testing and/or repeated open application test (ROAT). Fractions giving a positive reaction are refractionated and the new fractions obtained tested on the patient(s). These steps are repeated until a positive fraction that contains the allergen(s) is obtained. (b)

Bioassay-guided fractionation combined with structure-activity relationship analysis (SARs). The mixture is chemically fractionated and the chemical composition of the fractions analyzed. Potential allergens among the myriad of compounds composing the fractions are selected by SARs analysis as reference compounds and tested on the patient(s) in order to determine the culprit(s)

3 Bioassay-Guided Fractionation Combined with SARs

The principle behind structure-activity relationships (SARs) is that properties of a chemical, with respect to its mode of interaction with a defined system, are inherent in its molecular structure. SARs are developed by looking for links between structure and biological activity, based on mechanistic or empirical grounds. In other words, the biological activity of a compound is seen as a function of the structural and physico-chemical properties of the chemical.

Progress in the understanding of the molecular basis of allergic contact dermatitis has helped the development of SARs for sensitizing chemicals. The first step in the induction of contact allergy to a chemical is the formation of a covalent, irreversible, bond between the sensitizer and skin proteins to form an antigenic complex. This occurs principally through a nucleophile-electrophile mechanism, as the majority of allergens contain in their chemical structure an electrophilic functional group able to react with nucleophilic amino

allergic contact dermatitis can be carried out. This is especially useful when patients also react to individual fragrance chemicals. The analysis can show the presence or absence of such chemicals in the oils. Gas chromatography-mass spectrometry (GC-MS) is an appropriate useful tool used to elucidate allergies to essential oils as they are volatile odorous mixtures of organic compounds. A very extensive analytical investigation by GC-MS has been reported on several essential oils used by aroma therapists who reacted to many of these oils [16]. One patient was also allergic to geraniol, linalool, linalyl acetate, and α -pinene and the other to geraniol, caryophyllene, and α -pinene. α -Pinene was present in 42 oil samples, linalool in 22, geraniol in 16, and caryophyllene in 37. Concentrations of these chemicals ranged in between 1 and 10% (v/v) in some oils and in few others >10%. Another example is the case of a patient that became sensitized from topical application of tea tree oil for therapeutic reasons [17]. GC-MS analysis identified the presence, among many other compounds, of 1,8-cineole, being the patient also positive to this compound.

acids in proteins [18]. It can also occur in some cases through radical mechanisms [19]. It is therefore reasonable to conclude that if a chemical is capable of reacting with a protein, either directly or after a metabolism (prohaptens) or oxidation step (prehaptens), then it has the potential to be a contact allergen. Nowadays there is considerable knowledge on the chemical functions present in

chemicals that are related to a sensitizing potential. This knowledge, relating chemical structures to skin sensitization, is decomposed in a series of chemical rules, also called “structural alerts.” A “structural alert” consists of a total or partial chemical structure known to present a risk. The presence of a “structural alert” in a structure is thus an indication of a capacity to modify skin proteins and thus behave as a skin sensitizer. Examples of “structural alerts” are shown in Table 1.

In the course of investigations on fragrance chemical allergy, a new approach for the identification of fragrance sensitizers present in complex mixtures was developed based on the combination of bioassay-guided chemical fractionation, chemical analysis, and SARs studies. The need to develop this methodology, complementing simple bioassay-guided fractionation, became apparent due to the difficulty to obtain more and more refined fractions in the case of complex fragrance mixtures. Basically, the fragrance mixture is chemically fractionated, and the fractions obtained are tested on the patient(s) allergic to the mixture by patch testing and/or by ROAT. Positive fractions are subjected to an extensive chemical analysis (GC-MS, LC-MS) in order to identify chemical composition and molecule structures. From the structures, a SARs analysis permits to select molecules with a suspected sensitizing potential as defined by the presence of structural alerts. These molecules (reference compounds) are then directly tested on the patient(s) for the identification of the actual sensitizer(s) (Fig. 1b). The major advantage of this approach is to avoid iterative fractionation/patient testing sessions that are time consuming, require considerable effort by the patients and the scientists involved, and very often are of low benefit. A practical example is the case of oak moss.

3.1 The Case of Oak Moss

Many natural extracts are used in the perfume industry. Natural extracts are complex mixtures containing several hundred different chemicals responsible for the complexity of the odor. And among them is oak moss absolute, prepared from

Table 1 Examples of “structural alerts” present in sensitizing compounds

Chemical function	Substituents	Structure
Aldehydes	R = alkyl, aryl	
Ketones	R, R ¹ = alkyl, aryl	
Aldehydes, amides, esters, and α,β-unsaturated ketones	R = H, C, N, O (not OH) R ¹ = not heteroatoms, esters, ketones R ² = not aryl except when R = H	
Phenyl esters	R = alkyl, aryl R ¹ = any	
Hydroquinones and their O-alkyl precursors	R = H, alkyl R ¹ = any	
Catechols and their O-alkyl precursors	R = H, alkyl R ¹ = any	
Anhydrides	R = any	
Aromatic primary and secondary amines	R = alkyl, aryl R ¹ = any	
Alkyl halides	R = alkyl X = Cl, Br, I	R—X

the lichen *Evernia prunastri* (L.) Arch. Because of its woody aroma and fixative properties, it has been extensively used in perfumery as a natural fragrance, particularly for masculine products. Oak moss is considered a major contact sensitizer. Included in FMI, it has been reported as the most common allergen among the FMI eight ingredients [20].

Oak moss absolute is prepared by extracting the harvested lichen with hydrocarbon solvents, subsequently treating of the so-called oak moss concrete with a mixture of alcohols. In the 1970s–1980s, the chemical composition of oak moss absolute was extensively studied, and the allergenic potential of the major components was evaluated [21, 22]. At that time, sensitivity to oak moss was associated with the presence of phenylbenzoates such as atranorin and evernic acid but also with usnic acid although not a phenyl benzoate. For many years benzene was used to prepare oak moss concrete. With time it was replaced by more polar hydrocarbons. The composition of oak moss absolute did then change, and even though several potential sensitizers had been identified from former benzene extracts, constituents of oak moss absolute obtained from the new extraction procedures and the allergenic status were not clear.

Bioassay-guided fractionation combined with SARs was applied to new oak moss absolute extracts [23]. Patients with previous known sensitivity to oak moss were recruited for the study. After a first round of fractionation of the natural extract by column chromatography and patch testing, attention was focused on the strongest eliciting fraction in the patients. Chemical analysis of this fraction by GC-MS identified eight chemicals derived from a resorcinol structure. SARs analysis indicated that they all should be considered potential sensitizers. In order to avoid too many patch tests, the most representative molecules covering the different molecular structures were selected and tested on the patients. Atranol and chloroatranol were this way identified as the major eliciting chemicals and methyl- β -orcinol carboxylate as

the minor one (Fig. 2a). Atranol and chloroatranol are degradation products formed during oak moss processing, after transesterification and decarboxylation of atranorin and chloroatranorin during the ethanolic treatment (Fig. 2b). Methyl- β -orcinol carboxylate, also obtained during this processing, is essentially responsible for the characteristic earthy-moss-like odor of oak moss products.

The results of this study allowed the identification of atranol and chloroatranol as offending chemicals in oak moss absolute. It was later shown that both atranol and chloroatranol elicit reactions at very low levels of exposure [24]. Differences in the elicitation capacity between both substances are counterbalanced by exposure being greater to atranol than to chloroatranol, as it has been found that oak moss absolute contains approximately 2.1% (w/w) of atranol and 0.9% (w/w) of chloroatranol [23]. Of both compounds, chloroatranol, a hitherto unknown fragrance allergen, was identified as the main allergen in oak moss absolute. Further exposure assessment together with dose-response elicitation studies showed that chloroatranol elicited reactions by repeated open exposure at the ppm level (0.0005%) and at the ppb level on patch testing (50% of patients elicited at 0.000015%) [25, 26]. These results were described as unprecedented.

As a consequence, the International Fragrance Association (IFRA) standard on oak moss extracts fixed the maximum concentration allowed in skin contact cosmetic products to 0.1%. Since 2008 there is also an IFRA restriction on the concentration of atranol and chloroatranol in oak moss extracts which must not exceed 100 ppm each [27]. In 2004, the Scientific Committee on Consumer Products (SCCP) recommended that atranol and chloroatranol should not be present in cosmetic products [28]. Sensitization data on treated oak moss with removed atranol and chloroatranol suggested that it was possible to reduce the content of both to <2 ppm each. Thus, a cosmetic product containing 0.1% oak moss would contain so low levels of atranol/chloroatranol that the risk of sensitization of

Fig. 2 Bioassay-guided fractionation-SARs study on oak moss. **(a)** Gas chromatogram of the strongest eliciting fraction (F4), together with chemical structures identified and patch test results of representative molecules and sub-fractions (courtesy Pr An Goossens). In the patch tests, 132/134 corresponds to a mixture chloroatranol 4/atranol 5 and 196 to methyl-β-orceinol carboxylate 6. Subfractions

F4c/F4d contained atranol and chloroatranol, whereas sub-fraction F4b contained methyl-β-orceinol carboxylate. Sub-fractions patch tests results were thus in agreement with patch test results of selected molecules. **(b)** Formation of atranol and chloroatranol from atranorin and chloroatranorin during oak moss processing

allergic reactions would be low. However, clinical testing was asked by the SCCP in order to demonstrate a reduction in the elicitation capacity

[29]. It was shown that treated oak moss was able to elicit positive reactions by patch test and repeated open application test (ROAT) studies in

previously sensitized individuals [30, 31]. The European Union has recently published regulation 2017/1410 finally prohibiting the use of atranol and chloroatranol in consumer products [32]. From 23 August 2019, cosmetic products containing those substances shall not be placed on the Union market, and from 23 August 2021, cosmetic products containing those substances shall not be made available on the Union market.

4 In Chemico Methods Based on Chemical Reactivity

Validated *in chemico* methods have not yet been successfully applied for the evaluation of hazardous components in complex mixtures, being this a missing component in the battery of recommended methods in risk assessment of potential skin sensitizers.

During this decade, considerable efforts have been made to improve this aspect essentially based on nuclear magnetic resonance (NMR) and fluorescence trap methods using cysteamine derivatives as model of nucleophiles in the skin. The NMR-dansyl cysteamine (NMR-DCYA) and the high-throughput screening-DCYA (HTS-DCYA) methods are *in chemico* approaches recently reported to assess the ability of potential skin sensitizers to covalently bind to DCYA, a model dansyl thiol. DCYA operates as surrogate of nucleophilic residues of skin proteins to study the ability of the test molecule to initiate haptenation events. The use of DCYA as nucleophilic agent enables the trapping of electrophilic and other thiol-reacting species. In the NMR-DCYA assay, the degree of electrophilic depletion over time is quantified using proton NMR techniques [33]. Thus, quantification is carried out assessing the depletion of the sensitizer, in contrast to validated methods based on the depletion of the nucleophile. Also, it is possible to have structural information and elucidate reaction mechanisms and the sensitizer site of reaction. The HTS-DCYA method enables a quick and sensitive detection of electrophilic compounds in a high-throughput screening using fluorescence assays [34]. The fluorescence thiol DCYA is incubated with the test

molecule and activated to initiate a covalent binding. The fluorescence response of the DCYA adducts is quantified using fluorescence end-point readings. The amount of reacting species is reported as a reactive index (RI), which correlates with the amount of DCYA adducts found after elimination of unreacted DCYA. Quantification of the total fluorescent emission can be thus used to estimate the total formation of DCYA adducts or reacted species. A drawback of these methods is that they only consider identification of reactive electrophilic sensitizers and do not consider sensitizers thought to modify skin proteins through the intervention of reactive radical intermediates [19].

4.1 The Case of Aged Tea Tree Oil

Essential oils are widely used by consumers in a broad variety of goods. Because of the high frequency of contact, essential oils and complex mixtures of chemicals are common causes of contact allergy. In a literature search, 79 essential oils were found to cause positive patch test reactions or allergic contact dermatitis [2]. Tea tree oil (TTO), together with turpentine oil, has been one of the sole essential oils for which large-scale patch testing has been carried out with single components. However, toxicological profiles are very difficult to extrapolate from the mere sum of the profiles of the individual constituents as required today. The properties of the oil are deeply affected by the presence and abundance of its components. To add more complexity, many constituents often undergo chemical changes over time and form degradation products. For example, monoterpenes are well known to form oxidized by-products upon air exposure. These processes usually occur via free radical mechanisms forming reactive organic radicals, hydroperoxides, endoperoxides, and also electrophilic products that can themselves even degrade.

The assessment of the sensitization potential of TTO is thus a typical example of the challenges that toxicologists encounter in the safety evaluation of essential oils. TTO is the volatile oil obtained by steam distillation from leaves and terminal

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branchlets of *Melaleuca alternifolia*, *Melaleuca linariifolia*, or *Melaleuca dissitiflora* [35]. It is a colorless to pale yellow liquid with a terpeny, coniferous, and minty-camphoraceous odor. It has been reported to have multiple biological activities such as anti-inflammatory, antitumoral, analgesic, antimicrobial, and biocidal, among others. However, of all essential oils, TTO has caused most allergic reactions since the first case reports were published in the 1990s from Australia, where the oil is produced. Still, TTO is classified as a moderate sensitizer in the local lymph node assay, chemical components lack “structural alerts” to act as skin sensitizers, and most constituents have been classified as potential pre-/pro-haptens [36–38]. However, TTO stored in open bottles or in a bottle opened several times suffers an aging process resulting in photooxidation that leads to degradation products, which are strong sensitizers. Air exposure leads to a threefold increase in the sensitization potency of TTO. A number of different culprits have been suggested, but the identification of the real sensitizers continues to be an arduous task. The most important sensitizers in aged TTO appear to be terpinolene, ascaridole, and α -terpinene and its oxidation products, 1,2,4-trihydroxymenthane, α -phellandrene, *d*-limonene, and myrcene. Other chemicals, albeit less frequent, are aromadendrene, *d*-carvone, *l*-carvone, terpinen-4-ol, viridiflorene, sabinene, *p*-cymene, and 1,8-cineole.

Several aged “so-named” TTOs have been considered to test the applicability of the HTS-DCYA method in a recent study [39]. Initially, changes in the chemical composition of the oils after aging were analyzed by GC-MS. The aged samples showed an increase in the amount of *p*-cymene, considered as an indicative marker for aging together with the formation of numerous peroxide derivatives. For all “authentic” TTOs, a tendency of increased reactivity with increased amounts of *p*-cymene was observed, even if *p*-cymene was found to be negative and nonreactive in the HTS-DCYA assay. In parallel, those with higher peroxide content resulted in the highest reactive index (RI). The oxidized TTO sample giving the higher RI value in the HTS-DCYA assay was further analyzed by ultrahigh-performance liquid chromatography coupled with diode array detection and mass

spectrometry (UHPLC-DAD-MS) in order to get more information in regard to the potential reaction products, the reactive species, and thus the potential sensitizers. As a result, the major DCYA adducts were isolated and the structures confirmed by classical nuclear magnetic resonance spectroscopy (NMR) sequences and/or mass spectrometry. The global approach of the work methodology is shown in Fig. 3. Isolation of compound(s) giving the major fluorescent peak demonstrated the formation of a mixture of two diastereomers (m/z 436) resulting from a 1,4-Michael addition of DCYA on 4-hydroxy-4-methylcyclohex-2-en-1-one (Fig. 4). This small electrophilic α,β -unsaturated ketone of m/z 126 corresponds to a reported degradation by-product of ascaridole [39]. Ascaridole is an endoperoxide derived from autoxidation of α -terpinene. It has been described that easy cleavage of the O-O bond leads to the loss of stable isopropyl radicals through alkoxyl radical intermediates (iPr $\cdot$) forming this way reactive 4-hydroxy-4-methylcyclohex-2-en-1-one [40]. Additional chromatographic peaks with associated m/z 478 were also observed. Based on the mass fragmentation pattern, adducts could have originated from isoascaridole via the nucleophilic thiol ring opening of the oxirane moiety. Indeed, isoascaridole is known for a long time now as a by-product obtained after decomposition of ascaridole [41]. In conclusion, α -terpinene may be one of the critical components that undergo singlet oxygen addition to give stable endoperoxides that can further form elusive species such as the α,β -unsaturated ketone intermediate. Presence of this kind of intermediates could be responsible for the observed concomitant irritancy and co-sensitization with increased concentrations of ascaridole and ascaridole-containing essential oils [42].

This study thus demonstrates that formation of electrophilic species via radical degradations is one of the possible pathways that could explain peptide reactivity and sensitization to aged TTOs. Assuming that a lot of skin sensitizers are electrophilic compounds reacting with nucleophilic amino acids in the skin, this study exemplifies how the HTS-DCYA assay could be used to analyze complex mixtures for the presence of stable or unstable

Fig. 3 Global approach for the identification of potential sensitizers in aged tea tree oil using the HTS-DCYA assay [39]

reactive electrophilic compounds as a proof of concept.

4.2 The Case of a Chamomile Plant Extract: Combination with Bio-guided Fractionation Using In Vitro Assays

Despite its reputation as a medicinal plant, allergic reactions to topically applied products containing chamomile extracts are frequent [43]. In a recent study, the use of *in chemico* methods NMR-DCYA and HTS-DCYA has been combined with bio-guided fractionation using the KeratinoSensTM assay to elucidate the culprit of the sensitizing potential of German chamomile extract (German CE) [44].

Initially, the ethanolic extract of German CE was chromatographically subfractionated. Only one of the subfractions was positive at the KeratinoSensTM assay showing luciferase induction without cytotoxicity. It was thus subsequently fractionated to isolate

the major constituents to be tested for skin sensitization potential. Among the constituents, polyacetylene tonghaosu and cinnamic acid derivatives *E* and *Z*-glucopyranosyloxy-4-methoxycinnamic acid contained structural alerts for potential skin sensitization (Fig. 5). To complement the KeratinoSensTM data, the NMR-DCYA and HTS-DCYA reactivity assays were then used. Surprisingly, these compounds were only weakly reactive in the HTS-DCYA assay with RI values lower to the positive control cinnamaldehyde. Natural polyacetylenes are known to be potential sensitizers that are unstable to air and light [45]. Similarly, tonghaosu was found to quickly degrade. Thus, a fraction enriched with tonghaosu was tested in the HTS-DCYA assay after aging, and an increased value of RI was obtained. Further, reactivity experiments were carried out using the NMR-DCYA assay. From NMR results tonghaosu was confirmed to be nonreactive with DCYA, whereas the aged fraction was found to contain a component clearly reactive with DCYA. Purification of the aged fraction identified this component as 1,6-dioxaspiro

Fig. 4 Possible reaction scheme for the formation of DCYA adducts with electrophilic and thus potential sensitizing compounds present in TTO

Fig. 5 Structures of isolated compounds from GermanCE and mechanism proposed for the formation of DNE from tonghaosu aging

[4.4]non-3-en-2-one (DNE) (Fig. 5). This was confirmed by comparison with a synthetically prepared sample, and its sensitization potential was further

confirmed using the LLNA. From the mechanistic point of view, the oxidation of tonghaosu to DNE

probably occurs through olefin photo-oxygenation by singlet oxygen via dioxetane formation.

Lastly, the applicability of the HTS-DCYA begins today also to be explored for the evaluation of skin sensitization of complex botanicals [46].

5 Conclusion

The opening step for any prevention strategy for skin sensitization is hazard identification. This stresses the necessity to have reliable methods for the prospective identification of new chemicals that possess the potential to cause skin sensitization, ideally before they are put into the market but also already present in consumer products. To complicate things, among all the ingredients present in consumer products, many are complex chemical mixtures such as essential oils or botanical natural extracts that may also have sensitizing properties. Today, the identification of chemicals culprit of the sensitizing potential of very complex chemical mixtures continues to be a puzzle even if during the last years a great deal of progress has been accomplished.

The present chapter is a brief overview of the methods existing today that allow the identification of sensitizers in those complex mixtures. Simple bioassay-guided fractionation is an established method from many years. Also, the methodology based on the combination of bioassay-guided chemical fractionation, patch test and/or use testing of individuals with fractions, detailed chemical composition analysis of positive fractions, and chemical SARs analysis of identified molecules is now a valuable tool for the identification of allergens in fragrance complex mixtures. The case of the natural extract oak moss absolute is detailed to illustrate the methodology. In addition, this last decade an important consortium of researchers are bringing new fresh air with methods based on chemical reactivity and fluorescence trapping, basically studying natural extracts after aging and air oxidation such as the example of tea tree oil. However, assessing the sensitizing potential of complex mixtures together with the identification of the culprits in the mixtures is still far from being unraveled. Moreover, following the identification of new allergens, it is critical to further conduct a

complete skin sensitization risk assessment based on ingredient exposure, allergenic potency, and dose-response studies. The importance of exposure, dose-response correlation, and potency estimation has been underlined in here for the found oak moss offending allergens, atranol, and chloroatranol.

Still, even if lots of successful efforts have been done, more are nevertheless necessary in order to establish a strategy that will allow assessing mixtures extracted from natural sources and their active sensitizing components.

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