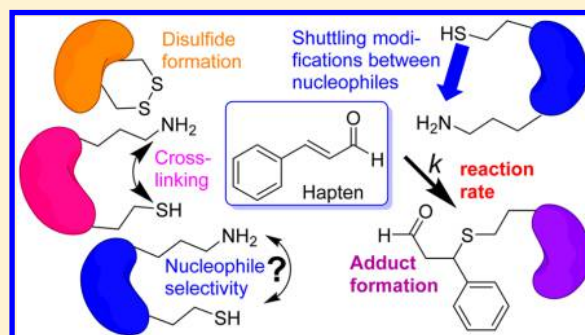


Reaction Chemistry to Characterize the Molecular Initiating Event in Skin Sensitization: A Journey to Be Continued

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ABSTRACT: The publication by the OECD of the adverse outcome pathway (AOP) for skin sensitization has accelerated the development and validation of mechanistic tests and testing strategies to assess the potential of new molecules to trigger skin allergies. The molecular initiating event (MIE) in the AOP is reaction with skin peptides/proteins. It is followed by a number of cellular events. Currently, only one *in chemico* test to characterize the MIE was proposed to and underwent adoption by the OECD, while two cell-based assays have completed the process. A multitude of further cellular assays is currently in the validation pipeline, but no further reactivity test has gone through full standardization. Here, we review data on *in chemico* methods, identify gaps, and discuss how these methods can be improved to better characterize the MIE and to become even more informative. We focus on the importance of kinetics, the information gained from studying adduct formation, and the difficulties posed by side reactions such as peptide oxidation. We then highlight mechanistic insights from reaction chemistry: the relative contribution of different target nucleophiles, the migration of amino acid modifications, and the potential of peptide-cross-linking. We illustrate in a case study how kinetic *in chemico* methods might have been used to better predict the risk of three preservatives, which have led to serious epidemics of contact dermatitis. In a case study on Michael acceptors, we show the impact of additional substituents around the electrophilic olefin moiety on reactivity, and we highlight the shortcomings which current *in silico* methods to predict reaction chemistry still have, illustrating the need for experimental *in chemico* data to improve such models. Finally, based on the information reviewed and the presented case studies, a strong argument is made to continue the journey of developing nonredundant, informative *in chemico* methods, and not to solely focus on new cell-based methods to further populate the AOP for skin sensitization.



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1. INTRODUCTION AND OVERVIEW

Skin sensitization is an immunological response against self-proteins, which have been chemically modified by exogenous chemicals in such a way that they are no longer tolerated by the skin immune system. This concept is widely accepted, and it is founded on the original observation on the importance of chemical reactivity for a molecule to act as skin sensitizer by Landsteiner and Jacobs 80 years ago.¹

Thus, skin sensitization starts with a chemical event, i.e., the reaction of a chemical with a protein or peptide, which is then followed by a number of biological events. These events include activation of different cutaneous cells, emigration of Langerhans cells transporting the modified peptide out of the skin, presentation of the modified peptides containing the novel epitopes in the lymph node, and finally lymphocyte proliferation and their secondary activation on repeated contact with the allergen. The whole process was summarized in an OECD document describing it as an AOP.²

Over the last 10 years, intensive research has focused on a more detailed understanding of both the chemical and the biological events. This renewed interest was not only motivated by scientific curiosity, it was greatly accelerated by regulatory pressure and the resources hence made available to come up with test systems to predict the sensitization potential of novel chemicals without the use of test animals. This research has led to the proposal of many new assays to achieve the goal of animal-free testing by both academia and industry. Several assays were taken up by validation authorities, and an accelerated process of validation led to three novel OECD guidelines for two cell-based biological assays (KeratinSens and h-Clat)^{3,4} and one *in chemico* assay, the direct peptide reactivity assay (DPRA).⁵ This is the first *in chemico* assay ever reaching regulatory acceptance.

If we consider the importance of chemical processes as the molecular initiating event (MIE) in skin sensitization, which are only followed by secondary biological processes, a certain bias can be observed in the assay development over the past few years: Currently, an ever increasing number of biological assays to predict skin sensitization is being proposed at the level of the validation authorities such as the European Center for the Validation of Alternative Methods to animal testing (ECVAM) and the organization for economic co-operation and development (OECD). At least six new biological assays (GARD,⁶ SENSIS,⁷ IL-8 luc,⁸ U-Sens,⁹ RHE IL18,¹⁰ and LuSens¹¹) were added to the ECVAM and/or OECD validation pipeline.^{12,13} The validation of improved reactivity tests currently does not keep pace with the development of new biological assay systems. This may be due to various factors, such as a higher interest in the subject from the biological community or larger funding made available for buzzwords such as “omics”, “pathway toxicology”, and “21st century toxicology”. However, given the importance of the reaction with a skin protein as the molecular initiating event, this bias may not be fully justified from a mechanistic viewpoint.

In this perspective, we will therefore review the work that led to regulatory acceptance of the DPRA and some recently proposed improvements to the DPRA to increase the information gained on the MIE, specifically focusing on reaction kinetics, adduct characterization, and unwanted side reactions. We then discuss the importance of different target nucleophiles, the possible significance of the transfer of haptenic modifications, and the ability of chemicals to cross-link proteins. We discuss a critical and somewhat controversial question: The proposal of *reactivity as rate limiting step*; is it sufficient to characterize reaction chemistry to make predictions, or, in other words, will each reactive molecule be able to sensitize, which would indicate that only the mechanism and kinetics of the chemical reaction need to be studied and a full coverage of the AOP may not be necessary?

We then present case studies to discuss open questions and give a perspective of how a more focused work on reaction chemistry may further advance the field: Could a stronger focus on reactivity improve the prevention of allergic contact dermatitis (ACD)? Case study on three epidemics to preservatives is given. Could a more detailed assessment of reaction chemistry improve *in silico* models? Shortcomings of the current *in silico* systems are highlighted by reactivity data.

Looking at all of the additional information, which may be gained from a molecular and quantitative characterization of the MIE, we argue that the journey for test development to harness the information from reaction chemistry studies is clearly not complete.

2. REACTIVITY TESTS TO CHARACTERIZE THE MIE OF SKIN SENSITIZATION: A BRIEF HISTORY

Landsteiner and Jacobs^{1,14} already observed in their 1935 and 1936 papers that highly reactive chemicals can cause sensitization in guinea pigs, while close analogues without reactivity are nonsensitizing. A clear correlation between the sensitization potential of nitrobenzenes and their ability to react with aniline was noted. Already in these seminal papers the authors proposed that “in the animal a reaction takes place by which the substances are converted into antigens. How and with what substances, proteins or others, a combination of the active compounds occurs, remains to be ascertained”. They further showed that animals previously sensitized to a chemical reacted to serum which was modified by the same chemical. Reactions included skin reactions or anaphylaxis, depending on the site of application. These experiments gave credibility to stable associations of the chemical with proteins in the serum used for elicitation as the ultimate antigen. Already in this seminal paper, the specificity of the immunological reaction was proven by cross-sensitization tests between analogues of increasing structural similarity. Thus, the stage was set and the basis of the hapten concept introduced: A stable association of a reactive chemical with a protein can trigger specific sensitivity toward this particular chemical. The hapten is the protein-reactive species, while the epitope is the stable immunogenic modification formed by the hapten on the target protein. The epitope is then recognized by T cells when bound to MHC molecules.

Since these original observations, reactivity tests have been extensively used. Initially, small model nucleophiles were used such as aniline in the Landsteiner study and later, e.g., butylamine and propylthiol mimicking key reactive amino acid side chains (see, e.g., refs 15 and 16). A number of studies used single amino acids, which were often protected by acetylation at the N-terminus to make the side chain the sole reactive group (see, e.g., ref 16). Several studies used glutathione (GSH) as a

simple model peptide: reactivity with GSH had already been a key focus in the toxicology of reactive molecules and especially reactive metabolites in other fields. Later, around 2000–2005, the focus shifted to studies with short synthetic model peptides and in fewer instances with whole proteins. In 2003, a peptide reactivity assay was proposed which is based on adduct formation with glutathione as detected by LC/MS.¹⁷ At the same time, a synthetic peptide combining all potential reactive residues was introduced and used in a peptide reactivity assay with HPLC for monitoring peptide depletion and adduct formation, followed by preparative HPLC and NMR for structure elucidation of selected adducts.^{18,19} The same peptide was later used by the group of Professor Karlberg in a number of mechanistic studies on adduct formation using LC/MS.^{20,21} A comprehensive review of different reactivity assays up to 2007 was compiled at an ECVAM workshop,²² and this summary will not be further reiterated here. Focus will rather be on the more recent studies after this review.

Increased interest by multiple groups in assessing peptide reactivity was triggered by the publication of the DPRA in 2004²³ and by a follow up publication with a refined protocol and prediction model including a large database on chemicals tested.²⁴ Most studies reviewed herein and data presented will therefore refer to reaction chemistry with model nucleophiles, mainly peptides. It should be noted, however, that a completely new approach to study reaction chemistry was presented recently by the introduction of high-resolution magic angle spinning NMR (HR-NMR MAS) to study the interaction of C13 labeled sensitizers with the proteome in a 3D-epidermis model.²⁵ This approach allows one to identify target amino acids for haptation but not to identify the modified protein. Another approach is to search for preferential target sites for modifications by reactive chemicals in the full proteome. One study using this approach with fluorescein isothiocyanate as model skin sensitizer was recently published.²⁶ Several proteomic studies identified reactive sites modified by different model electrophiles and both environmental and endogenous electrophiles.^{26–28} Such studies will shed light on the question of how a specific skin sensitizer interacts with proteins in the environment of the skin. It is tempting to use these methods to search for true immunogenic modifications under *in vivo* conditions. However, the most quantitative modifications must not necessarily be the most immunogenic, which would only be identified by characterizing the binding target of dominant T-cell clones in sensitized animals/individuals. However, due to technical complexity it is unlikely that these new proteomic approaches soon will become part of the assessment of new chemicals.

3. DIRECT PEPTIDE REACTIVITY ASSAY OECD TG 442C: THE GOLD STANDARD

The DPRA is a pragmatic assay: it measures the depletion of two test peptides, one with a lysine (Lys-peptide) and one with a cysteine residue (Cys-peptide) as key reactive site, after at least 24 h of incubation of the peptides with an excess of test chemicals. It is based on HPLC-UV quantification of the peptides containing phenylalanine as a chromophore. This highly quantitative and reproducible readout is coupled to an easily interpretable prediction model: chemicals with an average depletion >6.38% are rated as potential sensitizers. This assay proved to be easily transferable to other laboratories and thus allowed a full validation, finally leading to OECD acceptance⁵ and implementation by regulatory authorities.²⁹

The advantage of the assay is clearly the accuracy of the readout and the technical simplicity; it gives a very rapid indication of the intrinsic reactivity of a chemical. It has a high sensitivity for direct acting haptens (sensitizers not requiring activation to become reactive),²⁴ but it also detects a number of prehaptenes which undergo spontaneous oxidation under the assay conditions to become reactive.³⁰ Finally, since the readout is a simple numeric value, which translates into a dichotomistic separation of sensitizers and nonsensitizers, but also into different reactivity classes,²⁴ the results are easily interpretable by the regulatory community with a limited education to interpret more complex data on chemical reactivity. Hazard identification, i.e., separation of sensitizers and nonsensitizers, was the key target evaluated in the validation studies, and the assay was mainly optimized for this purpose. Indeed, a good predictivity of the DPRA was found^{24,31,32} (accuracy vs local lymph node assay (LLNA) 75%–89% depending on the data set).

However, following the famous saying that everything should be “as simple as possible, but no simpler”, there are a few reasons to question whether the information provided by these two depletion values are sufficient for all purposes. The implementation of the DPRA thus triggered a number of studies on modification of the assay by several laboratories, addressing potential shortcomings, which will be discussed in detail below:

- limited dynamic range due to lack of truly kinetic data;
- no information on peptide-adduct;
- no discrimination of adduct formation from side reactions such as peptide oxidation;
- single target nucleophile at one time, excluding effects of migration of chemical modifications or cross-linking of different residues;
- and lack of metabolic activity.

4. POTENTIAL REFINEMENTS OF THE DPRA

4.1. Kinetics: Improved Dynamic Range. According to the DPRA test guideline, HPLC analysis of DPRA samples starts at 24 h after test setup, but since triplicate samples of up to 15 chemicals are tested in parallel and many control samples are included, a typical analytical sequence may last over 24 h. Therefore, the result recorded is depletion anytime between 24 and 48 h and basically reflects an end point or equilibrium rather than a defined point in a kinetic reaction, which was never the goal when the DPRA was originally developed. Interestingly, this has limited effect on the accuracy of the readout with little variation between triplicates, but it indicates that the result does not fully reflect how fast the test chemical reacts. At the same time, only one concentration of the test chemical is tested (10-fold excess for Cys- and 50-fold excess for Lys-peptide). Therefore, the readout is 0–100% depletion at completion of the reaction at one test dose. Assuming that the results reflect the true 24 h value and that we can accurately distinguish between 5% and 95% depletion from background variation, we can transfer this range to kinetic rate constants according eq 1.³³

$$k \text{ (s}^{-1}\text{M}^{-1}\text{)} = \frac{\text{LN}\left(\frac{100}{100 - \text{depletion}}\right)}{(86400\text{s} \times 0.005\text{M})} \quad (1)$$

This leads to rate constants from 1.2×10^{-4} to 6.9×10^{-3} ($\text{s}^{-1}\text{M}^{-1}$) for reaction with the Cys-peptide, thus covering less than 2 orders of magnitude. Measured rate constants for extreme sensitizers with the Cys-peptide in the range of $0.2\text{--}3 \times 10^0 \text{ s}^{-1}\text{M}^{-1}$ were reported.³⁴ From this follows that the DPRA

approach thus has two limitations: (i) the dynamic range is much lower than the potency spread measured in the LLNA (over 4 orders of magnitude); (ii) we have no idea whether the measured time point is still in the linear range of the reaction.

This has been discussed in detail by theoretical publications from Professor Roberts and co-workers³⁵ and subsequently tested experimentally in our laboratory.^{33,34,36} All these kinetic studies were based on the principle of peptide depletion as the final readout (with the associated shortcomings discussed below), but they included multiple doses and/or multiple time points. A kinetic analysis of reactivity with the Cys-peptide has been applied to sensitizers in defined applicability domains, showing a linear relationship between rate constants and potency in the LLNA within certain domains;^{33,34} see Figure 1

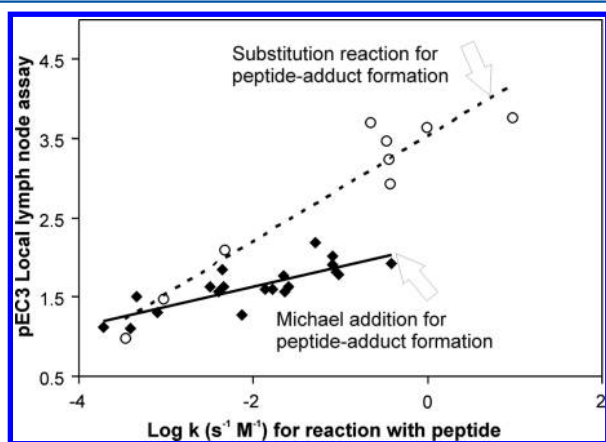


Figure 1. Relationship between potency in the LLNA and kinetic rate constants for Cys-peptide depletion for sensitizers in two specific mechanistic domains: Chemicals reacting by Michael addition (closed diamonds) and chemicals reacting by addition/elimination (open circles). An anti-inflammatory action correlating to the reactivity was shown for the Michael-acceptors, but not for chemicals in the other domain. The anti-inflammatory action could reduce the danger signal provided by these chemicals under the test conditions of the LLNA, thus dampening their sensitization potency. Reprinted from ref 34. Copyright 2011 American Chemical Society.

reproduced from previous work.³⁴ Interestingly, the association between kinetic rate constants and sensitization in the LLNA was weaker (smaller slope of the regression) for Michael acceptors than for sensitizers reacting by addition/elimination. We proposed as a possible explanation the known anti-inflammatory action of reactive Michael acceptors which may act as a dampening factor, at least in the setting of the LLNA, where the test chemical must provide both antigenicity/novel epitopes and a secondary inflammatory signal.

Improved prediction of potency with kinetic data was also found in a more global analysis on 244 chemicals.³⁶ The correlation between logarithmic LLNA potency and the logarithmic rate constant derived from a 24 h depletion value is $r^2 = 43.6\%$, and it is improved to 51.7% by using a kinetic assessment. This analysis also showed that global sensitizer potency as measured by the LLNA is only partly explained by reactivity data from this type of assay alone.³⁶

These studies indicated the added value of kinetic data, and more examples will be shown in the case studies below. However, one key issue is not resolved: kinetic analysis of depletion reflects the sum of all reactions taking place and is not necessarily representative of adduct formation (see below).

Nevertheless, different approaches are available to measure kinetics of peptide depletion, and overall, only minor changes to the principle setup of the DPRA are needed to generate these additional data, which would make such changes to the original assay easy to validate.

4.2. Characterization of Adduct Formation. Since the MIE consists of the stable association between the chemical and a peptide/protein, it can be argued that directly monitoring the formation of these adducts is the most direct assessment of the allergenic potential of a chemical. Very detailed studies on few selected sensitizers have been performed over the last 20 years by the group of Professor Lepoittevin, using the experimental approach of synthesizing C^{13} labeled haptens and following their reaction with nucleophiles of increasing molecular complexity to determine in detail the structure of the formed adducts and potential reaction mechanisms and pathways.^{37–41}

In order to screen a larger number of molecules and to avoid the need for labeled molecules, LC/MS has been used by different groups to detect adduct formation with peptides. With this approach, it is easy to determine at which nucleophilic site of the peptide a reaction takes place, and the molecular weight of the modification can be determined. If the reaction is more complex than just a direct addition or a Schiff base formation, the exact nature of the novel bond cannot be easily elucidated. However, it is possible to combine preparative HPLC with NMR in selected cases of adducts with unexplained molecular weights, and a detailed structure analysis of peptide adducts then becomes possible without labeled haptens.^{18,19,42} As summarized above, it has become fashionable to measure reactivity with peptides as the closest surrogate of the unknown *in vivo* target. However, similar reactions often will take place with simple nucleophiles such as propylthiol and butylamine. For structure elucidation by NMR, such simple adducts are easier to handle, thus it should be emphasized that some successful projects on specific adduct formation have used such simple nucleophiles,⁴³ sometimes in parallel with more complex peptides.

It had been discussed whether preference should be given to measuring peptide depletion over analyzing adducts, and the argument was made that the depletion end point is the better choice for screening.³⁷ However, this does not have to be an either/or decision, as both outcomes can routinely be screened with LC/MS in the same experiment.^{44,45}

4.3. Specific Published Case Studies on Adduct Formation. The group of Professor Lepoittevin compared the two preservatives 2-methylisothiazol-3-one (MI) 1 and 5-chloro-2-methylisothiazol-3-one (MCI) 2. Overall, similar reaction pathways were observed when testing small nucleophiles or amino acids and GSH:^{16,40} MI was shown to react exclusively with thiols, while the extreme sensitizer MCI could react both with thiols and with amines (see Figure 2). Interestingly, thiols such as GSH or N-Ac-Cys-OMe were able to activate MCI to become particularly reactive with amine groups.⁴¹ Subsequent studies could confirm similar reactions both under the conditions of the DPRA,³⁷ in intact proteins⁴¹ and in a 3D-skin tissue using highly advanced NMR-magic angle spinning analysis.⁴⁶

Skin-sensitizing hydroperoxides were another focus of studies on adduct formation over recent years: Specific adducts with radicals generated from limonene-2-OOH with GSH and N-Ac-Cys-OMe⁴⁷ and of radicals derived from linalool-OOH with N-Ac-His-OMe were described.⁴⁸ On the basis of LC/MS analysis, a number of adducts between these hydroperoxides and N-Ac-Cys-OMe, N-Ac-Lys-OMe, and N-Ac-Trp-OMe were detected, along with radical-catalyzed oxidation of N-Ac-Trp-OMe and

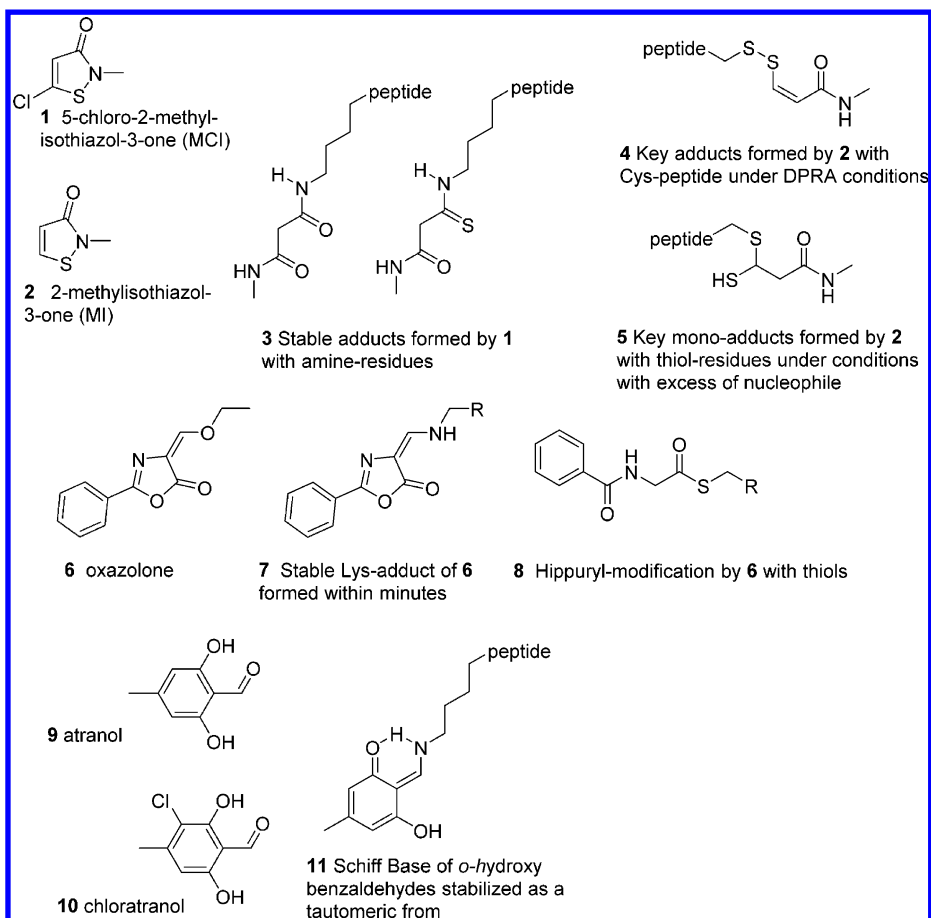


Figure 2. Specific adducts observed in case studies on potent sensitizers.

N-Ac-Tyr-OMe.⁴⁹ Adduct formation with highly reactive $\alpha\beta$ -epoxy oximes was studied in detail to elucidate reactivity at different nucleophilic sites in a model peptide comprising all potential nucleophilic residues.²⁰

We have performed two case studies on some of the most extreme sensitizers known. Similar to the studies on MCI, we thought that focusing on extreme cases may illustrate some of the key reasons for a chemical to have a particularly high sensitization potential. Oxazolone **6** is the most potent sensitizer according to the LLNA. Adduct formation with both the Cys- and the Lys-peptide was studied in a kinetic manner.⁵⁰ We found 100% Cys-peptide depletion after 20 min. However, the formed Michael-addition adduct was unstable leading to peptide regeneration, and a different, more stable, thio-acyl adduct **8** formed during the 24 h reaction leading to only partial peptide depletion at the end of the reaction. More striking was a specific and very rapid Michael addition followed by ethanol elimination with amine residues (56% depletion after only ca. 3 min). The corresponding adduct **7** was highly stable. We concluded that this rapid and stable association with amine residues may be a key reason for the extreme sensitization potential.⁵⁰

In a similar study on different benzaldehydes, we again compared adduct formation to the sensitization potential.⁴² One key outcome of the study was that simple aldehydes and benzaldehyde could form Schiff bases but that these are rarely detectable under classical DPRA conditions due to rapid hydrolysis in an aqueous environment. This probably also happens in the body, and it is difficult to imagine how Schiff bases formed at the topical application site can migrate to the immunological tissue in the lymph node

without being hydrolyzed in the lymphatic fluid, unless they are somehow protected against hydrolysis. Nevertheless, stable adducts did accumulate in these experiments: surprisingly, abiotic oxidation of the unstable Schiff base led to low quantities of stable amides.

More interesting, however, was the case of *o*-hydroxy benzaldehydes such as atranol **9** and chloratranol **10**. These chemicals were long present as contaminants in oak moss extracts used mainly in male fine perfumery. A median level in commercial perfumes of 0.5 ppm was reported,⁵¹ but this level appeared sufficient to trigger significant numbers of allergic reactions. This epidemiological evidence led to legal limits in Europe. The elicitation potency of these sensitizers was tested in a repeated open application test (ROAT) and in dose-response patch test studies: Concentrations in the low ppm range (0.1–4 ppm) could trigger reaction in 50% of sensitized individuals.^{52–55} These are probably the lowest elicitation thresholds ever reported. LC/MS analysis indicated that **9** and **10**, unlike most other aldehydes, formed stable adducts with amine groups under aqueous conditions with a molecular weight consistent with a Schiff base. More detailed analysis using NMR then showed that not the direct Schiff base but its tautomeric form **11**, forming a stable H-bond, is present in water, explaining the unusual stability of this adduct. We concluded that this highly stable tautomeric Schiff base **11** with amine residues is the reason for the unusually high sensitization potential of *o*-hydroxy benzaldehydes and that this is a substructure which should be generally avoided in the design of new molecules. These case studies on adduct formation are summarized in Figure 2, and

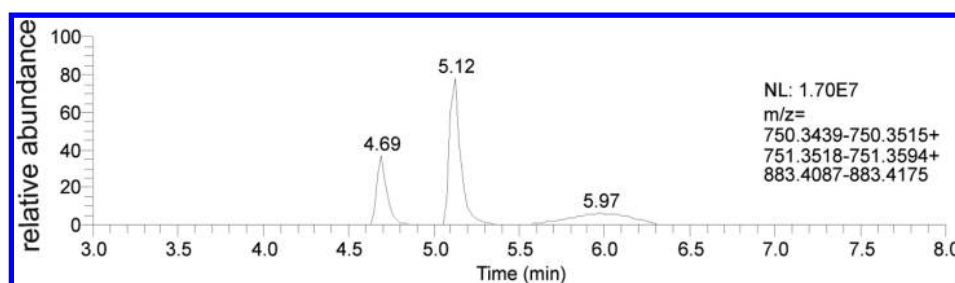


Figure 3. LC/MS analysis of a positive control (cinnamic aldehyde) sample in DPRA performed according OECD guideline 442c. The sample containing some white precipitate was homogenized by ultrasonication, diluted 1:100 in H₂O, and analyzed by high resolution LC/MS. Shown is the sum of the extracted exact mass traces of (i) the parent peptide (monitored as the [M+H]⁺ ion; *m/z* = 751.3556), (ii) the peptide dimer (monitored as the [M+2H]²⁺ ion; *m/z* = 750.3477), and (iii) the hypothetical direct Michael adduct (monitored as the [M+H]⁺ ion; *m/z* = 883.4131). The parent peptide elutes at 4.67 min (depletion vs control 81.4%), while the dimer elutes at 5.13 min, and the peak of the direct adduct appears at 6.0 min.

Table 1. Different Scenarios on the Effect of Oxidative Side Reactions on the Overall Interpretation of Peptide Reactivity Tests

scenario	adduct formation early in reaction	adduct formation at 24 h	peptide oxidation early in reaction	peptide oxidation at 24 h	conclusion for hazard ID	conclusion for rate constant/quantitative readout
A	(+)	+	–	–	unambiguously positive	Depletion rate constant reflects true reactivity.
B	+	+	+	+	unambiguously positive	Depletion rate constants may only partly reflect true reactivity.
C	+	–	–	+	Positive, but early adduct formation needs to be recognized.	Depletion rate measured early reflects true reactivity.
D	–	–	(+)	+	Potential false-positive, if no transient adduct formed	Depletion rate overestimates the true reactivity.

they clearly indicate that we can still learn something new on structural alerts by studying reaction chemistry.

4.4. Side Reactions: Peptide Dimerization and Oxidation. When first implementing the DPRA in our lab and starting to monitor the reaction not only by the HPLC-UV-based peptide depletion readout but also by LC/MS, we noted that a number of chemicals triggered Cys-peptide depletion in the absence of any specific adduct formation. The reaction mixture rather contained a white precipitate which mainly consisted of the disulfide formed by oxidative dimerization of the test peptide.⁵⁶ This observation was confirmed by other research groups,^{30,44} and a similar observation was also made later in a project on reactivity with GSH in a different research field.⁵⁷ This side reaction happens quite frequently, and it may be important to understand it better in order to properly interpret the DPRA result from a molecular standpoint. We thus list here a few observations made: (i) peptide dimerization is often triggered by aldehydes; so far, we did not observe concomitant reduction of the aldehyde; hence, we currently cannot explain the molecular mechanism. (ii) Peptide dimerization is sometimes triggered by chemicals without any structural alert for sensitization/reactivity. (iii) Peptide dimerization often occurs in parallel to adduct formation, and the observed depletion is a sum of both processes. This is, for example, the case for the positive control in DPRA, cinnamic aldehyde as shown in Figure 3. (iv) In some cases, dimerization appears to be a secondary reaction after initial formation of an unstable adduct, as we show in the case study on preservatives below. In this case, depletion due to dimerization indeed is indicating sensitization potential. Thus, the two processes may but do not necessarily have to be causally linked. (v) The process is not strongly dependent on the test peptide. We have tested a number of chemicals both with the Cys-peptide and with the Cor1-C420 peptide used in the LC/MS based assay.⁴⁵ For several chemicals, unspecific dimerization is observed for both peptides but not always. There are also chemicals without a structural alert which

catalyze dimerization of only one peptide, adding a stochastic element to the problem. (vi) As indicated, in the case of the Cys-peptide, the dimer has very poor solubility in the test medium and cannot be quantified under the HPLC-UV conditions according to the test guidelines. Quantification is possible after homogenizing the samples by ultrasonication, followed by a 100-fold dilution in water and analysis by sensitive LC/MS. In the case of the Cor1-peptide, the peptide dimer is soluble and gives sharp peaks under standard conditions, thus facilitating quantification.

On the basis of these observations, multiple scenarios are possible as summarized in Table 1. They indicate that it is not always easy to conclude on the true reactivity of a chemical. This is particularly true if dimerization becomes the dominant process.

Thus for hazard identification, i.e., to decide whether a chemical indeed is reactive, depletion in the absence of adduct formation may lead to a false-positive result (scenario D). However, this is not crystal clear, as the absence of adduct formation at the end of the reaction does not exclude that transient adducts were present earlier (scenario C). In addition, if dimerization is very fast, it may completely consume the peptide, preventing any slowly forming adduct from appearing. Finally, there are obviously many “false-corrects”; the DPRA correctly predicts sensitization potential for aldehydes, e.g., but the result is not due to observed adduct formation under the experimental conditions but rather due to the side reaction. Whether indeed in all these cases peptide oxidation is a consequence of transient adduct formation is not obvious.

The case becomes even more complex for rate constant determination based on peptide depletion and its application to the assessment of sensitizer potency. Since we often observe both reactions for some chemicals but only adduct formation for others, a chemical able to trigger both processes may be estimated to be more reactive even if in reality this is not the case. Hence, better separating these processes will not only improve

accuracy in hazard identification but also help to better assess sensitizer potency.

One approach is presented in the case studies on preservatives. Distinguishing the two processes by a kinetic analysis of DPRA samples by LC/MS over time may help to identify the dominant reaction in questionable cases. Another approach very recently proposed used simple nucleophiles (butylamine and butylthiol) and measured kinetics of adduct formation and test chemical depletion by NMR.⁴³ Reaction conditions were adapted for each chemical in order for the reaction to proceed significantly over a 1 h experiment. Under these conditions, thiol-dimerization was only a minor reaction, and true kinetic rate constants for adduct formation were determined. So far, it was not tested whether this approach yields rate constants that are indeed better correlated to sensitizer potency as compared to values based on peptide depletion, but one would theoretically assume that this is the case.

Finally, it should be noted that next to oxidation by dimerization, also oxidation of the thiol group to the sulfone (+32) or sulfoxide (+48) is observed;^{44,45} again, some chemicals catalyze this process by an unexplained mechanism leading to peptide depletion in parallel to or in the absence of adduct formation.

4.5. Addition of Metabolic Capacity to Reactivity Tests.

Some sensitizers need activation, mainly by either abiotic (prehaptens) or enzymatic (prohaptens) oxidation. While the former reaction appears to take place for some chemicals under the incubation conditions of a typical peptide reactivity experiment,^{30,58} enzymatic oxidation relevant for the conditions in the skin is more difficult to mimic. In a modification of the DPRA, a simple surrogate oxidative system is added consisting of hydrogen peroxide and horse radish peroxidase. This system activates chemicals with an intrinsic potential for (enzymatic) oxidation, leading to peptide depletion only in the presence of the oxidative system.⁵⁹ Successful detection of prohaptens by this approach was shown. In drug discovery, metabolic activation by liver microsomes and trapping of the reactive metabolites with a nucleophilic species (mainly GSH) is routinely performed. This approach was also applied to the skin sensitization field. When using liver enzymes, it may be oversensitive from a predictive toxicology standpoint, as also terpenes without sensitization potential were activated at the double bond to form epoxides binding to GSH.⁶⁰ Nevertheless, it is certainly a good screening approach for potential metabolic activation to reactive species. Alternatively, liver microsomes can be added to peptide reactivity tests and formation of adducts between synthetic peptides and reactive metabolites assessed. However, we found only a small group of sensitizers, mainly phenols such as eugenol, activated in this test.

5. MECHANISTIC ASPECTS WHICH CAN BE INVESTIGATED WITH REACTION CHEMISTRY

5.1. How Does Reactivity with Different Nucleophilic Residues Affect Sensitizer Potency? There has been a debate on which amino acid residues in proteins are the most important targets for sensitizers.⁴⁴ Cysteine is the most reactive residue toward the majority of known sensitizers, and therefore, reactivity with cysteine appears the most sensitive end point from a predictive viewpoint, with a majority of sensitizers showing a preferential Cys-reactivity. This is especially true for weak sensitizers. Statistical evaluations integrating cysteine and lysine reactivity results on a large number of chemicals attributed a higher importance to cysteine reactivity compared to lysine

reactivity when modeling potency of the universe of known sensitizers,^{31,61,62} but on a smaller set of chemicals also, the opposite result was found.⁴⁴ Preferential reactivity with cysteine was repeatedly observed in a peptide containing all potential nucleophilic side chains (PHCKRM) when testing benzoquinones,¹⁸ $\alpha\beta$ -epoxy oximes,²⁰ and reactive epoxides.²¹ On the other hand, there are sensitizers for which stable modifications of lysine residues only were observed, e.g., acid anhydrides.

However, strong and extreme sensitizers were often less selective and able to react with multiple residues under experimental conditions (high test chemical concentrations and/or elevated pH). Can we derive from this observation, that a strong sensitizer derives its potency from the fact that it is able to target multiple residues in proteins hence generating various different epitopes and thus triggering a stronger immune response? This may be an intellectual shortcut: There are indeed sensitizers with a specific molecular mode of action, reacting specifically with other amino acids than just cysteine, and this may partly explain their potency as highlighted below. However, in other cases an extremely reactive chemical may be observed to react with less reactive sites in a protein under experimental conditions with high concentrations of test chemicals, but this is just due to the fact that we can force the reaction *in vitro*. For example, very strong Michael acceptors can react with lysine residues but preferentially react with cysteine. Now consider how potency is measured *in vivo*: the true measure of potency is the dose per area triggering sensitization.⁶³ An extreme sensitizer needs a 1000-fold lower dose as compared to a weak sensitizer to induce sensitization, and in a simple view, this means that a 1000-fold lower dose will generate a sufficient number of novel epitopes to pass the threshold for sensitization. This 1000-fold lower dose may suffice to modify an adequate number of the most reactive residues, which are cysteine residues even if the chemical has been observed to be able to modify lysine or even tyrosine residues under selected reaction conditions *in vitro*. Thus reactivity with multiple residues *in vitro* may just be an indicator of very strong reactivity and will not always inform us on the true target residues at the lowest inducing concentration *in vivo*, which determines sensitizer potency.

However, there are exceptions in which reactivity with a specific amino acid may be the key to understanding potency. A unique case is the correlation of corticosteroid sensitizer potency with the ability of their degradation products, containing a glyoxal substructure, to react with arginine.⁶⁴ Furthermore, in the three case studies on reaction chemistry of the very powerful sensitizers MCI, (chlor)atranol, and oxazolone summarized above, a specific reactivity with lysine residues and formation of stable amine-adducts is a common theme (see Figure 2). Thus, modification of the abundant free amino groups in cells may indeed be a particularly strong immunogenic signal.

A further interesting case is 3,4-dihydrocoumarin **12**. It is a moderate sensitizer in the LLNA (EC₃ = 5.6%). In two human studies, it induced allergy in 25 of 25 or 32 of 62 of individuals with an inducing dose of 20% and elicitation at 20 and 2%, respectively.⁶⁵ This was certainly a high test concentration, but the rather high frequency of induction in human individuals indicates a high allergenic potential. **12** has an exclusive reactivity vs amino groups, leading to 40% Lys-peptide depletion when tested at pH10.^{44,66} However, only <5% of the Lys-residues are modified when tested at physiological pH (our unpublished observation). Thus, relatively low reactivity toward lysine under physiological conditions translates into a significant sensitization

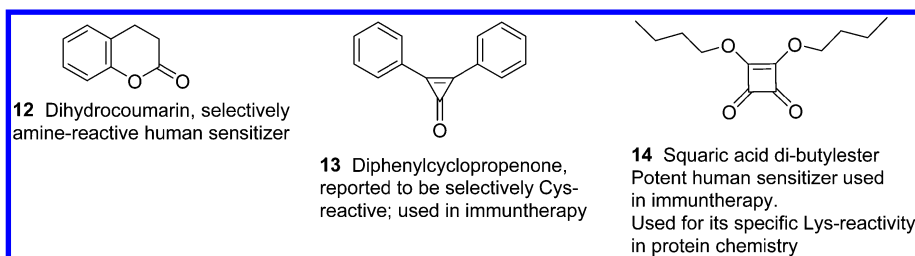


Figure 4. Human sensitizers with unique reactivity vs Cys- or Lys-residues. Note: the Lys-reactivity of squaric acid dibutyl ester is known from protein chemistry but not observed under DPRA conditions.

potential. This case further illustrates the significant sensitization potential of amine-reactive agents.

In conclusion, high reactivity toward lysine can indicate (i) a general high reactivity or (ii) a specific mode of action. In both cases, Lys-reactivity may indicate a significant sensitization risk. Thus, specifically avoiding strongly amine-reactive chemicals in the discovery process of new ingredients may be a reasonable strategy.

However, it is also clear that the absence of Lys-reactivity and exclusive Cys-reactivity does not preclude a very powerful sensitization potential. MI 2 discussed in detail below is a clinically very strong human sensitizer although having an exclusive Cys-reactivity. Further illustration may come from diphenylcyclopropenone **13** and squaric acid dibutyl ester **14** (Figure 4): these chemicals are clinically used for local immunological therapy of warts due to their ability to reliably provoke strong allergic reactions in humans.⁶⁷ While the former was reported to exclusively react with Cys under DPRA conditions,⁶⁸ the latter specifically reacts with Lys-residues (a property exploited in protein chemistry⁶⁹), yet both are very strong human sensitizers.

5.2. Sequential Reactivity with Different Amino Acid Residues: Transfer of Peptide Modifications. One interesting aspect repeatedly discussed in specific cases but not yet systematically explored is the potential transfer of amino acid modifications from the original site of haptenation to other residues to form more stable adducts. This final target may then be the true immunogenic epitope and not the transient first modification. This may be especially relevant, if the transient proximal modification resides on an abundant cellular thiol, i.e., glutathione (or not yet discussed for skin sensitization, coenzyme A).

This brings us back to the case study on MCI mentioned above, where thiol groups have been shown to activate MCI to react more efficiently with amine groups; hence, glutathione could enhance the modification of serum albumin by MCI.⁴¹ However, it was hypothesized that this transfer happened not directly but with the formation of a free, highly reactive thioacyl chloride intermediate (Figure 5). Similarly, also in the reactivity studies of MI 2 with GSH, formation of a reactive intermediate after reaction with a first nucleophile was shown, which then can react with a secondary target. In the case study on oxazolone,⁵⁰ the thiol adduct accumulating after 24 h, thioester **8**, was shown to be able to transfer the modification both to another Cys- and (to a lesser extent) to the Lys-peptide. Hence, an initial modification of GSH can in principle be transferred to proteins not in direct contact with the sensitizing chemical **6**. Adducts between aromatic isocyanates with Cys-residues could be directly transferred to lysine residues to form more stable adducts.³⁸ In a study on allyl isothiocyanate,⁷⁰ a semistable Cys-adduct transferred the modification to a Lys-residue, thus forming a more stable thiourea derivative. Transfer of modifications generated by

di-isocyanatetoluene from GSH to a thiol residue in a peptide was also shown.⁷¹ Finally, transfer of an unstable modification on a Cys-residue by hexahydrophthalic anhydride to form stable modifications on Lys-residues was described.¹⁹ Sensitizers forming adducts which may be shuttled to other residues in such a way may need special attention. These literature cases are summarized in Figure 5.

5.3. Cross-Linking of Proteins. Another interesting subject, which can be addressed with more detailed reaction chemistry studies, is the ability of chemicals to cross-link multiple nucleophilic residues. If a chemical can cross-link multiple proteins or multiple residues within one protein, in theory a more complex antigenic structure may be formed. The ability to cross-link proteins is particularly important in respiratory sensitization, with di-isocyanates or dialdehydes such as glutaraldehyde or glyoxal (or their metabolic precursors) acting as potent respiratory allergens.⁷² In skin sensitization, the particularly strong sensitization potential of dialdehydes has been recognized too, and it was related to their ability for protein-cross-linking.⁷³ In the case studies on oxazolone and salicylaldehydes, we had recognized the presence of a second electrophilic center after the addition of the first nucleophile.^{42,50} However, under the peptide reactivity conditions no cross-linking had been observed in our earlier studies. We have reinvestigated this question here by incubating some strong sensitizers with an excess of propylthiol and/or butylamine. Our first results are shown in Figure 6. Oxazolone **6** can first react with either propanethiol or butylamine at the side chain by Michael addition under EtOH elimination and subsequently react at the ester-function to form thioesters **15** and **16**. Atranol **9** forms a stable Schiff-base with butylamine as described in Figure 2. As such, **9** is not reactive with thiols, and no adduct with propylthiol is detectable. However, after reaction with butylamine, a quinoid tautomeric structure is formed, and propylthiol can add to the butylamine adduct to finally form **17**, also indicating a certain cross-linking ability. However, this is only the minor reaction product even with an excess of propylthiol being present. Finally, MI can react with two propylthiol residues as previously observed to form **18**. In this case, the diadduct is equally abundant as the monoadduct **5** under our experimental conditions.

These results are only summarized here, and we will investigate and report on this subject further. However, these experiments indicate that in the three case studies on different powerful sensitizers summarized above, an additional cross-linking ability is a common theme. How important this ability is for the overall sensitization potency is currently unknown.

In respiratory sensitization, antibodies of the IgE type do play a role. It is possible that the more complex antigenic structures formed by some respiratory sensitizers with a protein cross-linking ability are able to preferentially induce the formation of antibodies, recognizing more complex haptenic determinants as

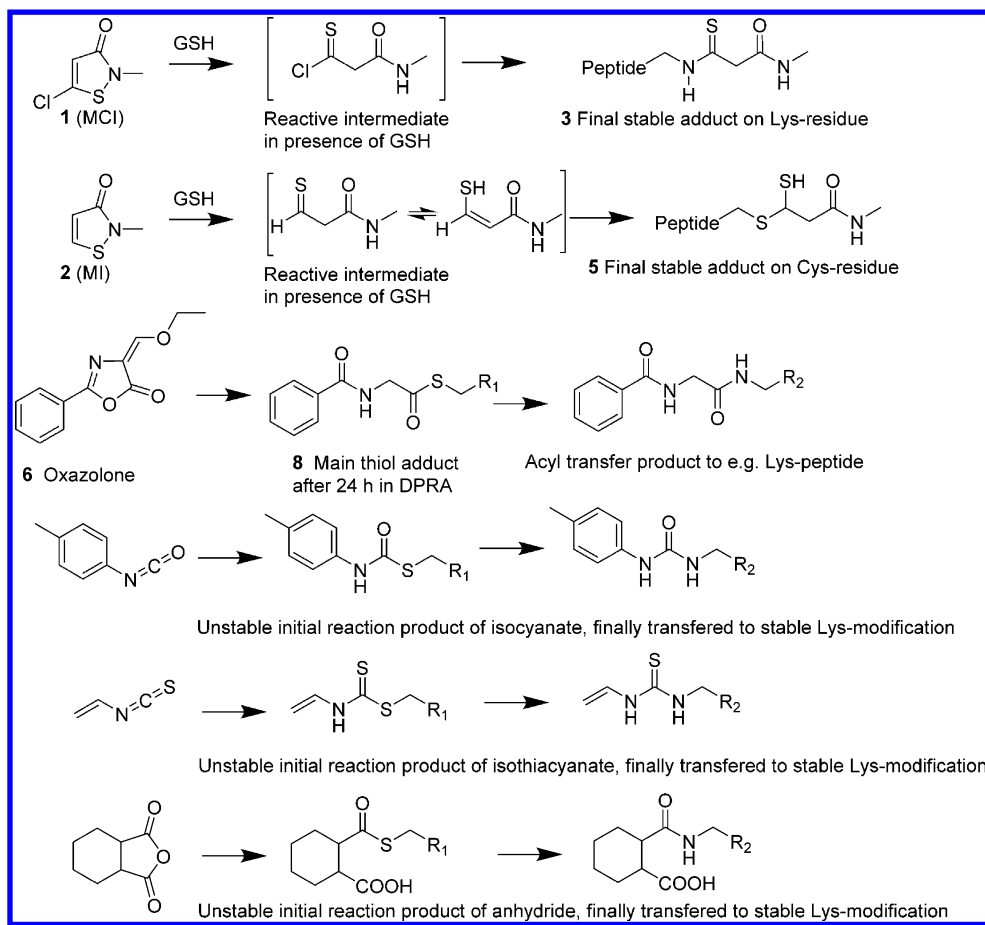


Figure 5. Literature cases of chemicals initially reacting with Cys-residues, followed by a shuttling of the modification to finally form stable modifications.

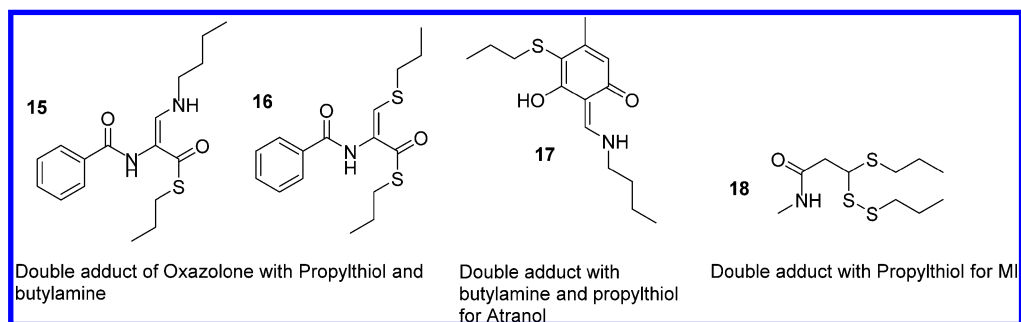


Figure 6. Double adducts, cross-linking two nucleophilic sites, formed by potent sensitizers incubated with an excess of propylthiol and butylamine. Note: These structures are based on the exact molecular mass determined with HR-LC/MS and the reaction pathways previously established. In the case of the atranol adduct, the exact site of the propylthiol addition on the benzylic ring is tentative and not yet fully confirmed.

compared to T-cells. Skin sensitization is primarily a T-cell mediated reaction; however, the possible implications of antibodies has recently been rediscussed.⁷⁴ The question may arise as to whether cross-linking chemicals are able to induce antibody formation in skin sensitization.

5.4. Proposal of Protein Reactivity as the Rate Limiting Step, or Will Each Reactive Molecule Be Able to Sensitize?

The concept of the AOP and its application to define integrated testing strategies based on multiple tests, each modeling individual key events, assumes that all the sequential steps in the process contribute to sensitizer potency. The AOP paradigm would indicate that we need to go all through the AOP and ideally characterize T-cell proliferation triggered by allergens; this is

clearly the final and most important key event in acquisition of sensitization.

However, there is the counter argument that the MIE is the rate limiting step; hence, adequately describing reaction chemistry will largely suffice to characterize the sensitization potential for a chemical.⁷³ Is this indeed the case? We can address this question from a statistical angle: when modeling LLNA potency data we found that indeed a kinetic rate constant was the most important single predictor but that predictions were further improved based on dose–response information from the cell-based KeratinoSens or h-Clat assays.³⁶ This result emphasizes the importance of reactivity but shows that additional information from cell-based assays contributes to potency prediction.

However, a mechanistic argument in favor of the simplistic view with a focus on the MIE comes from inspection of the sensitization potential of increasingly complex chemicals. Several alkylating agents which are able to transfer methyl groups are reported as sensitizers. Transfer of a methyl group is the smallest modification of a protein we can envisage, and obviously, it is sufficient for a vigorous T-cell reaction.⁷³ Thus, there appears to exist no lower boundary to the required molecular complexity of the epitope to trigger sufficient immunogenicity. From this observation, one may derive that indeed any reactive chemical is able to sensitize.

However, there may be one exception to this rule: protein modifications that are occurring within the body naturally may trigger a tolerance of the immune system, and reactive chemicals forming these same epitopes would then have no or at least a reduced ability to sensitize. Such an example may be the transfer of acetyl groups. In principle, all phenyl acetates should be able to sensitize due to acetyl transfer, but the transfer of acetyl groups is a process widely used in the body to modulate gene and protein activity; thus, it is possible that the body has a tolerance to acetyl-modification of proteins (although we have to keep in mind that enzyme-catalyzed acetylation happens only at specific residues). This would explain the negative LLNA result for 4-carboxyphenylacetate, which in our hand led to 77% peptide depletion due to acetyl transfer in the Cor1-C420 assay, and reactivity with both the Cys- and the Lys-peptide in DPRA was reported.³² (However, positive LLNA and GPMT had been reported for 4-sulphophenylacetate,⁷⁵ and we cannot exclude the possibility that 4-carboxyphenylacetate is false-negative in the LLNA, and indeed, also that acetyl-transfer is sufficient for sensitization to occur.) Whether such tolerance to “endogenous happens” really exists may be an interesting question. One may consider, e.g., ascorbic acid: oxidation and degradation products are known to react with proteins and glutathione,^{76,77} and ascorbic acid is positive in some *in vitro* assays, but it is clearly nonsensitizing in humans. Could this be due to intrinsic immunological tolerance toward modifications generated by its reactive degradation products, which are repeatedly present in the body (Figure 7)? A similar question relates to the endo-

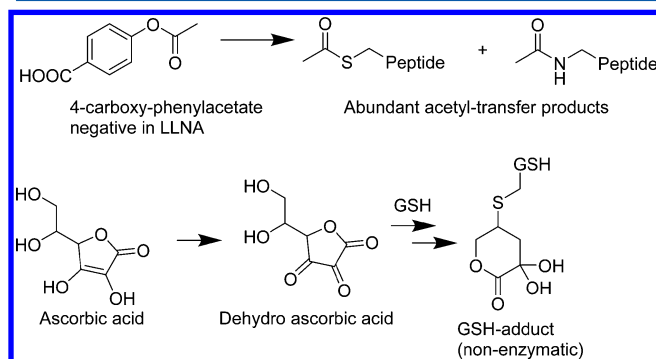


Figure 7. Peptide adducts which may be nonimmunogenic due to tolerance. Both 4-carboxy-phenylacetate and ascorbic acid are negative in the LLNA and are considered nonsensitizers despite their ability to modify peptides or GSH.

genous electrophiles like 4-hydroxynonenal and malondialdehyde. Protein modifications by these reactive lipid peroxidation products should be “known” to the immune system. Does the body therefore have an intrinsic tolerance to proteins haptenated by these electrophiles? Since they are not abundant in all tissues but rather under pathological condition of oxidative stress, this must, however, not be the case.

6. CASE STUDIES

6.1. Could a Better Understanding of Reaction Chemistry Improve the Prevention of ACD? Case Study on Three Epidemics to Preservatives. Over the last 25 years, significant epidemics of contact allergy toward preservatives in cosmetic products occurred. Methylidibromoglutaronitrile (MDBGN) became a frequent sensitizer at around 2000⁷⁸ before it was banned for leave-on products in 2005, while MI rapidly became the most prevalent contact allergen next to nickel between 2008 and 2015,⁷⁹ triggering a proposal to ban it for leave-on and rinse-off products. Iodopropinyl *N*-butylcarbamate (IPBC) was used less often, but in relation to its use, it is still recognized as a significant contact allergen⁸⁰ (for structures, see Figure 8).

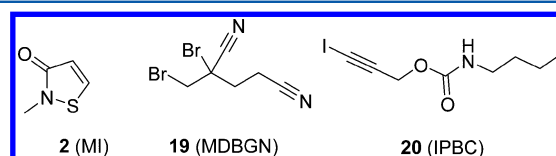


Figure 8. Structures of three preservatives causing significant epidemics of contact allergy over the last 25 years.

We did investigate the kinetics of the reactivity of MI in more detail. In our first publication on kinetic profiling in 2009,³³ we already showed that MI does react very fast with the DPRA Cys-peptide, with 95% depletion after 3 min. This result had been obtained with a fluorescent derivatization of the free thiol group. Here, we repeated this result with a kinetic LC/MS experiment under DPRA reaction conditions (see Figure 9 for MI 2 and damascenone as reference sensitizer). Indeed, after 5 min the parent peptide is no longer detectable and is transformed quantitatively to an adduct with a molecular weight of 865.6, which is consistent with ring opening and formation of a disulfide bridge (4 in Figure 2). The reaction rate with the Cys-peptide estimated in our earlier work is $>5.1 \text{ s}^{-1}\text{M}^{-1}$. With $>99\%$ depletion due to adduct formation at 5 min in the LC/MS experiment, we can confirm this rate constant for the direct reaction with the Cys-peptide to be $>3 \text{ s}^{-1}\text{M}^{-1}$. This is significantly higher as compared to the extreme sensitizer 2,4-dinitrochlorobenzene (DNCB) ($k = 0.22 \text{ s}^{-1}\text{M}^{-1}$).³⁴ Hence, the kinetic analysis of peptide reactivity indeed predicts a very strong sensitization potential for MI. In addition, MI has the ability to cross-link multiple sulfur groups in proteins (see above, Figure 6). Thus, if indeed protein cross-linking proves to be a relevant mechanism to increase the sensitization potential, this would further underline the sensitization potential of MI.

However, there is also another observation made when investigating the DPRA reaction for MI over time using LC/MS. The initially formed adduct appears not to be stable, and it is degraded $>50\%$ after 24 h under formation of the peptide dimer (Figure 9). Thus, the DPRA after 24 h indicates full depletion but only partial adduct formation. However, this may be due to the specific condition of the DPRA with the test chemical in excess. If the nucleophile is in excess, a different final adduct with MI is formed (5 in Figure 2).³⁷

We also performed the kinetic DPRA on the two other preservatives 19 and 20. Similarly to MI, both led to complete peptide depletion within 5 min. Thus, the rate constant based on depletion again is $>3 \text{ s}^{-1}\text{M}^{-1}$ and thus higher as compared to DNCB. However, in this case no adduct could be detected, but the peptide had completely dimerized within this short time

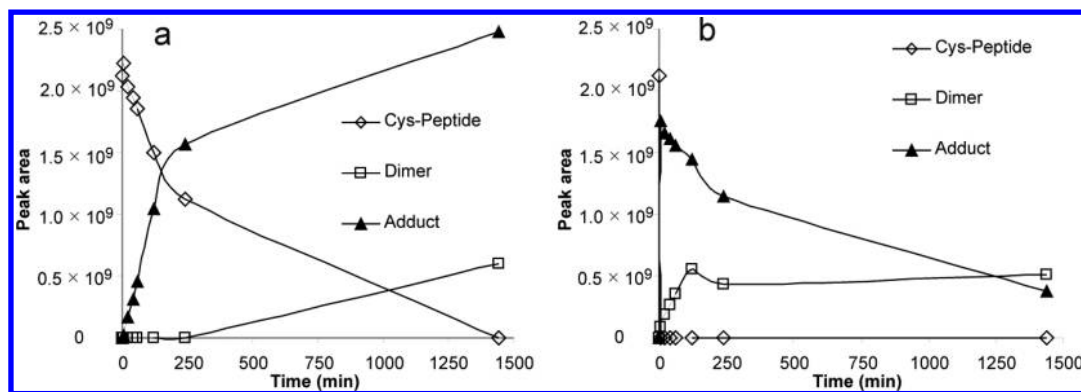


Figure 9. Kinetic analysis of DPRA samples with LC/MS. DPRA samples were prepared according to OECD TG 442c and then repeatedly injected in LC/MS. (a) Damascenone. Note the linear reaction of adduct formation over the first 4 h. Later some peptide dimerization occurs. (b) MI 2. Note instant complete peptide depletion and adduct formation within the first 5 min, followed by gradual loss of the adduct and formation of the peptide dimer. In this analysis, samples were not diluted, and the dimer precipitates from the solution, and the dissolved amount does not further build up after 2 h due to solubility limitations.

span. This is highly surprising and further underlines the difficulties posed by this side reaction discussed above. We assume that a highly unstable initial adduct is formed and that the peptide dimerization is a secondary event in these cases. The reaction was thus repeated with propylthiol or butylamine. Complete disappearance of the parent molecule for both preservatives in the presence of propylthiol, but not butylamine, was observed, but no adducts could be detected so far. Currently, we cannot fully explain the reactivity of these two preservatives and what type of stable protein modifications they may cause, explaining their sensitization potential at the molecular level. This is certainly a subject worthy of further investigation, in order to understand their strong human sensitization potential and the epidemics they caused. It may well be that their reaction chemistry cannot be adequately studied in solution but that rather an *in vivo* reactivity test in the complex structure of the cellular proteome is needed, e.g., using NMR-MAS.²⁵

However, what the three preservatives do have in common is the extremely fast reactivity with the Cys-peptide when measured as depletion rate constants, which exceeds the rate constants measured for most other strong or extreme sensitizers. Performing such kinetic measurements for new chemicals therefore is certainly extremely useful. It is always easy to argue with hindsight, but we can still perform the thought experiment: Given (i) our current understanding of the importance of the MIE in skin sensitization and (ii) assuming this extremely fast peptide reactivity, surpassing the reaction rate of extreme sensitizers such as DNCB had been known at the time of introduction of these preservatives into multiple cosmetic products in the mass market. Would the risk assessment have come to the same conclusions or would the cosmetic chemists have refrained from introducing such extremely reactive species into their formulations? Or, to say it in other words, would we again promote topical leave-on application of chemicals with such high reaction rates measured by *in chemico* assays? Developing preservatives which attain the goal of eliminating microbial contamination but which lack such high reactivity with peptides is certainly a key challenge ahead.

6.2. Reactivity Tests vs *in Silico* Models: Alert Accuracy.

Case Study on Michael Acceptors. Simple 2-D structural alerts, which are encoded in rule-based modeling software, are often used to predict sensitization potential. They do recognize a wide variety of potential sensitizers, but even simple rules may have significant shortcomings. An experimental assessment

of reaction chemistry may still be superior to a simple *in silico* analysis as illustrated here first with the case of ionones and damascones (also called rose ketones). The structures of four molecules along with their peptide reactivity (reaction rate with the Cys-peptide) and both human and animal sensitization data are summarized in Table 2. This Table also includes test results from the cell-based KeratinoSens assay, which indicates activation of the transcription factor Nrf2 by electrophilic chemicals. Damascones 21 and 22 are natural minor constituents of rose petals. When their importance for perfumery was discovered and they became synthetically available, a significant skin sensitization potential was soon recognized in predictive human tests. This led to a strict self-regulation of these ingredients by the industry.⁸¹ Ionones such as 23 on the other hand, have been widely used in perfumery, and they were tested in multiple tests, both in guinea pigs and in humans, indicating no significant sensitization potential (reviewed in refs 82–84). Methyl-ionone 24 had been tested at a very high dose (70,000 $\mu\text{g}/\text{cm}^2$) in a human repeat insult patch test (HRIPT), and no reactions were observed. This ingredient is listed on the 26 labeled allergens in the EU; however, a review on clinical patch test data⁸⁵ indicated that it has the second lowest frequency of positive reactions (0.11%) among the 26 labeled allergens, despite high use frequency (ranked fifth among the 26). Thus, the risk to become sensitized has been estimated as almost negligible.⁸⁵ As shown in Table 2, based on a potential Michael acceptor alert, all four presented molecules are predicted as strong sensitizers by the TIMES SS⁸⁶ model, which currently is probably the most advanced software for this purpose. However, adduct formation with the Cys-peptide (Table 2) indicates that the damascones are highly reactive molecules, while almost no reaction (ca. 1% adduct formation) is observed with β -ionone. With 24, no adduct formation could be demonstrated. For β -ionone, a possible explanation for the surprisingly low reactivity is a steric shielding of the β -position by the gem-dimethyl group. In the case of 24, the reactivity is probably further reduced due to branching in α -position. As summarized in Table 2, the rate constant for peptide reactivity, Nrf2-induction in KeratinoSens, and the human sensitization data are very well aligned, much better than the predictions from the *in silico* model. In the case of 24, the LLNA does not align with the human and reactivity data. This may be due to significant cytotoxicity/irritancy of that material, an effect which may lead to false-positives in the LLNA.

Table 2. Human, Animal, *in Vitro*, and *in Silico* Sensitization Potential and Reactivity of Damascones and Ionones

	Name	LLNA data (EC3 in %; class)	Human data (LOEL $\mu\text{g}/\text{cm}^2$) ¹	Cys-Reactivity ($10^3 \times k [\text{s}^{-1}\text{M}^{-1}]$)	Concentration for Nrf2 induction KeratinoSens (μM)	<i>In silico</i> prediction ²
	21 Damasconone	1.22; moderate	500	52.2	2.8	Strong ³
	22 β -Damascone	6.7 / 2.4; moderate	230 (cis isomer)	4.5	4.75	Strong ³
	23 β -Ionone	no data, non-sensitizing in 9 guinea pig studies	non-sensitizing in small HRIPT study	0.02 (ca. 1% adduct after 24 h)	>1000 ⁴	Strong ³
	24 Methyl- α -ionone	21.8; weak	>70866	No adduct	>1000	Strong ³

¹LOEL = lowest observed effect level for the induction of sensitization in a predictive human test. ²Prediction by TIMES SS software. ³TIMES SS output: "strong sensitizer; $\alpha\beta$ -carbonyl compound with polarized double bonds". ⁴Average induction, 1.42; positive in 3 of 7 experiments; this result indicates weak activation but is below the threshold for positive rating by a prediction model.

Table 3. Human, Animal, *in Vitro*, and *in Silico* Sensitization Potential and Reactivity of Some Michael Acceptors

	Name	LLNA data (EC3 in %; class)	Human data (LOEL $\mu\text{g}/\text{cm}^2$)	Cys-Reactivity ($10^3 \times k [\text{s}^{-1}\text{M}^{-1}]$)	Concentration for Nrf2 induction KeratinoSens (μM)	<i>In silico</i> prediction
	25 cinnamic aldehyde	1.3; ¹ moderate	775	29.1	16	Strong sens.; $\alpha\beta$ -aldehydes
	26 α -methyl-cinnamic aldehyde	4.5; moderate	>3540	0.3	41	Strong sens.; $\alpha\beta$ -aldehydes
	27 Isojasmonone	no LLNA data, very mild sensitizer in GPMT ²	no data	0.05 (ca. 2% adduct after 24 h)	144	Weak sens.; $\alpha\beta$ -carbonyl comp. with polarized double bonds
	28 2-hexylidene cyclopentanone ³	2.3; moderate	500	4.4	13	Weak sens.; $\alpha\beta$ -carbonyl comp. with polarized double bonds

¹Geometric mean from multiple tests in a database from Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ²GPMT with 5% intradermal, 25% topical induction: mild response at 10% elicitation. ³Note: all of the animal, human, and *in vitro* results indicate very similar sensitization potentials of **28** to β -damascone in Table 2.

We can further look into the effect of branching in α -position for the Michael acceptors in Table 3. Cinnamic aldehyde **25** and methyl-cinnamic aldehyde **26** are both predicted to be strong sensitizers by TIMES SS, but the human sensitization potential estimated by HRIPT differs by a factor >5, and the kinetics of Cys-peptide depletion differs even more (100-fold reduced for Michael addition; but of course, a theoretical ability to react with amine residues is still present in **26**). Again, the reactivity data predict a difference in the human sensitization not revealed by

the *in silico* predictions. A further discrepancy is illustrated for two jasmones: **28** was found to be a moderate sensitizer in both human and animal tests. On the basis of the *in vivo* data, Cys-reactivity, and the KeratinoSens result, the sensitization potential of **28** is indeed similar to that of β -damascone (Table 2). The related isomer **27** with the double bond in the ring, on the other hand, does form very low levels of Cys-peptide adducts (ca. 2%), it does induce luciferase in KeratinoSens only at 10-fold higher concentration, and was found only as very mild sensitizer in a

guinea pig maximization test (GPMT). Both molecules are predicted equal (weak sensitizers) by the modeling software, despite a dramatic difference in kinetic reactivity and sensitization potential. In this case, both molecules do have a branching in α -position. However, this is not sufficient to avoid reactivity in the case of **28**; nucleophile attack could be facilitated by hydrogen bonding between the substrate carbonyl and a sulfhydryl group, or alternatively, the release of conformational strain upon Michael addition may enhance reactivity of **28**, effects not fully understood and therefore not yet encoded in the *in silico* alerts. The data presented in this case study further underline the notion that both kinetic reactivity data and the concentration for luciferase induction in KeratinoSens can partly explain potency differences, at least when used within such mechanistic domains.

6.3. Reactivity Tests vs *in Silico* Models: Quantitative Prediction of Protein Binding. The examples given above show limitations of current *in silico* models to predict the effect of subtle modifications on reactivity, but often such modifications may be critical when designing new molecules. We will report shortly how such small modifications can be used to design damascenes with reduced sensitization potential. Here, we also performed a more global analysis. TIMES SS software gives a semiquantitative prediction of protein binding ("AmountAdduct/mol"). A very recent study built classification trees for the prediction of the skin sensitization hazard based on both *in silico* and measured *in chemico/in vitro* data.⁸⁷ "AmountAdduct/mol" was found to be the most predictive parameter, and models built upon it were reported of a higher predictivity as compared to models using measured data. This certainly is a challenging finding, especially for those active in lab work in *in vitro* toxicology and considering the high efforts made to develop and validate experimental *in chemico* and *in vitro* methods over the past decade. This very recent study thus made us curious to further test the "AmountAdduct/mol" predictor. We made a statistical comparison of the rate constants of peptide reactivity measured on a data set of 244 chemicals³⁶ versus this predictor. If we correlate measured logarithmic rate constants against logarithmic EC3 values from the LLNA, we find a correlation coefficient $r^2 = 0.5$ as indicated above. The correlation of log (AmountAdduct/mol) vs log EC3 is clearly lower ($r^2 = 0.32$ with TIMES-SS v 2.27.13 used by Asturiol et al., $r^2 = 0.35$ with recent version 2.27.17). Hence, at least when facing the problem of potency prediction, the quantitative measured value still appears superior, despite the finding of Asturiol et al. in their approach toward hazard assessment.

These comparisons with the modeling results are not made here for a "bashing of *in silico* approaches"; we regularly use the TIMES SS model for early predictions. The case is made here only to stress a key point of this perspective: following the road to get even better measured data cannot yet be replaced by modeling. The approaches are complementary and reinforce each other: findings such as the case studies in Tables 2 and 3 can ultimately be used to fine-tune and improve the *in silico* models.

7. OUTLOOK: THE DEVELOPMENT OF NEW OECD ACCEPTED METHODS

In the Introduction, we highlighted the broad array of new biological tests for skin sensitization addressing key events 2 and 3 in the AOP, which are currently in the validation pipeline. The key question that needs to be addressed in the near future is whether these tests add new information when added to existing integrated testing strategies, or whether they can replace existing

tests thereby increasing prediction accuracy. This question can only be addressed by large database-driven statistical evaluations. However, given that some tests address the same signaling pathways and based on the mechanistic links between these molecular pathways,⁸⁸ there is a high probability that the information gained from testing in multiple cellular systems does significantly overlap and that some tests generate largely redundant data.

In our eyes, the information summarized in this perspective demonstrates that further investments into peptide reactivity assays are clearly warranted to balance a more detailed coverage of the key events 2 and 3 obtained by these new tests with a more granular understanding of the molecular initiating event. This is particularly true since the MIE may be rate-limiting in many instances as discussed above. Ideally a more detailed test would still incorporate the readout of the DPRA (i.e., 24 h depletion) but also integrate additional information such as kinetics and characterization of reaction products. It was not the goal of the current work to present such a new assay, but it may be timely that 10 years after the ECVAM workshop,²² experts would convene and discuss such potential refinements.

Furthermore, we should not underestimate the molecular mechanistic nature of the information obtained from reactivity tests, which may further guide us on key structural alerts which should be avoided in new chemical development. This molecular understanding along with experimental screening tools will also help to find structural modifications to significantly reduce reactivity, which in turn will assist in the design of safer chemicals. This information can only partly be obtained from cell-based tests.

Finally, the insights obtained from studying strong and extreme sensitizers, both related to the molecular targets preferably modified and the kinetics of peptide modifications, may directly be used in risk assessment. As highlighted by the preservatives causing recent epidemics of contact allergy, an understanding of their reaction kinetics may have helped to make a more stringent risk assessment. While reactivity data summarized here today are still largely used in an "explanatory manner" (i.e., to explain what we already know from animal and clinical data), they may and should in the future be used in a more "predictive manner". This is particularly pertinent with the move away from animal-based testing.

8. EXPERIMENTAL PROCEDURES

Rate Constant Determinations. Rate constants with the Cys-peptide were determined as described before.³⁴ For chemicals with very low reactivity, i.e., chemicals not leading to significant peptide depletion, rate constants can be estimated from the amount of adduct formed according eq 1. However, this is only an estimation as it assumes the LC/MS response factor of the adduct to be similar to the parent peptide.

Kinetic DPRA Followed by LC/MS Analysis. Samples were prepared according to the standard DPRA protocol. They were then injected after 5, 20, 40, 60, 120, 240, and 1440 min on a Dionex UltiMate RS 3000 HPLC system coupled to a Thermo Velos pro ion trap mass spectrometer with electrospray ionization (ESI) in positive mode. The ion source was operated at 4 kV, the sheath gas flow rate was set to 35 arb, and the aux gas flow rate to 10 arb. The temperature of the capillary was kept at 275 °C. Mass spectra were recorded from m/z 500–1500. A ZORBAX Eclipse XDB-C18 column, 2.1 mm ID, 150 mm, and 5 μ m (Agilent Technologies) was used. Eluent A consists of water containing 0.1% formic acid, and eluent B consists of

methanol containing 0.1% formic acid. A gradient was run from 95% eluent A (hold for 2 min) to 60% eluent B within 2 min, to 98% eluent B within 8 min (held for 2 min), back to 95% eluent A within 1 min followed by 1 min equilibration time. The solvent flow rate was 250 $\mu\text{L}/\text{min}$. The injection volume of the sample was 2 μL .

Reactivity Assays with Propylthiol and Butylamine.

Reactivity tests were performed under DPRA conditions, in a 25% acetonitrile mixed with 75% phosphate buffer (50 mM, pH 7.5). Test chemicals at a final concentration of 0.5 mM were incubated with an excess (5 mM) of either propylthiol or butylamine or a combination of both nucleophiles. Samples were analyzed after 4 and 24 h with LC-HRMS analysis on a Dionex UltiMate XRS 3000 HPLC system coupled to a Q-Exactive orbitrap mass spectrometer (Thermo Scientific) with electrospray ionization (ESI) in both positive and negative ionization modes. For liquid chromatography separation, an XBridge C18 column with dimensions 2.1 mm $\times$ 50 mm and particle size of 2.5 μm with a 2.1 mm $\times$ 10 mm precolumn of the same material (Waters) was used. The flow rate was 200 $\mu\text{L}/\text{min}$. Eluent A consists of water containing 0.1% formic acid, and eluent B consists of methanol containing 0.1% formic acid. A linear gradient was run from 90% eluent A (hold for 1 min) to 100% eluent B within 6 min (hold for 1 min), back to 90% eluent A within 1 min, followed by 1 min equilibration time. The injection volume of the sample was 1 μL . The resolution of the HR-MS spectra was set to 70,000. The mass accuracy was <5 ppm. Data-dependent high resolution product ion spectra (HR-MS/MS) were recorded at a resolution of 17,500. Ion source parameters adjusted were as follows: sheath gas flow (35 arbitrary unit), auxiliary gas flow (10 arbitrary unit), capillary temperature (300 $^{\circ}\text{C}$), and source voltage (4 kV in positive mode; -3 kV in negative mode). Fragmentation was obtained from dissociation in an octopole collision cell using higher energy collision dissociation settings at 35 and 45 (arbitrary unit). The mass scan range was set from 80 to 1200 m/z .

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Notes

The authors declare no competing financial interest.

Biographies

Andreas Natsch got his PhD from Swiss federal Institute of Technology in environmental microbiology in 1996. He moved to the research department of the Fragrance manufacturer Givaudan in 1998. First key research focused was the elucidation of the biochemical mechanisms of human body odor formation to design new deodorant ingredients. Since 2008, the research focus has moved to the development and implementation of *in vitro* assays to study toxicological risks associated with cosmetic and fragrance ingredients without using animal tests. He now leads the *in vitro* toxicology lab to evaluate risks of novel fragrance ingredients early in the development pipeline.

Roger Emter got his PhD from the University of Basel in biochemistry in 2005. He joined Givaudan in 2006 where he first focused on biochemical studies on odor releasing enzymes. He then developed the KeratinoSens cell line and set up the KeratinoSens assay, which was adopted as OECD guideline in 2015. Currently he works in the development of new assay systems and application of the existing tests in the screening of novel ingredients in the development pipeline.

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ABBREVIATIONS

ACD, allergic contact dermatitis; AOP, Adverse Outcome Pathway; DNCB, 2,4-dinitrochlorobenzene; DPRA, direct peptide reactivity assay; ECVAM, European Center for the Validation of Alternative Methods to animal testing; GSH, glutathione; HRIPT, Human Repeated Insult Patch Test; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; IPBC, iodopropylbutylcarbamate; LLNA, local lymph node assay; EC3, concentration for 3-fold stimulation of cell division in the draining lymph node in the LLNA; LOEL, lowest observed effect level; MI, 2-methylisothiazol-3-one; MCI, 5-chloro-2-methylisothiazol-3-one; MDBGN, methylidibromoglutaronitrile; MIE, molecular initiating event; Nrf2, nuclear factor-erythroid 2-related factor 2; OECD, Organization for Economic Co-operation and Development; QRA, quantitative risk assessment; ROAT, repeated open application test

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