

Review Article

Regulatory classification of substances oxidized to skin sensitizers on exposure to air

ANN-THERESE KARLBERG¹, DAVID BASKETTER², AN GOOSSENS³ AND JEAN-PIERRE LEPOITTEVIN⁴

¹National Institute for Working Life, Department of Occupational Medicine, Dermatology Division, S-171 84 Solna, Sweden

²Unilever SEAC Toxicology, Unilever Research, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK

³Katholieke Universiteit Leuven, University Hospital, Department of Dermatology, B-3000 Leuven, Belgium

⁴Université Louis Pasteur, Clinique Dermatologique, Laboratoire de Dermatochimie associé au CNRS, F-67091 Strasbourg Cedex, France

ESCD Working Party on the Chemical Basis of Allergic Contact Dermatitis

Regulatory classification of substances in the European Union (EU) is intended to identify their hazardous toxicological properties in a formal and harmonized manner. In the regulatory work, a specific chemical with its molecular structure is classified as a skin sensitizer. This implies that the compound is stable throughout its lifetime. The purpose of the present paper is to discuss the problem of skin sensitizing oxidation/degradation products formed by air exposure of various materials or substances with very low allergenic activity. In regulatory classification work on skin sensitizers, the intrinsic susceptibility of a chemical to air oxidation (autoxidation) should be taken into consideration. We give examples of natural terpenoid materials, but the concept of allergens formed by air oxidation can apply to other materials widely used in industrial products. If a positive classification is made for a substance with a known chemical structure, a note should indicate that the primary chemical structure of the notified substance is not a skin sensitizer, but that (some of) its oxidation products are. Complex mixtures which inevitably contain sensitizing oxidation products should (on the basis of sufficient evidence) be classified as skin sensitizing.

Key words: regulatory classification; skin sensitizers; air oxidation; contact allergy; prohaptens; limonene; colophony; legislation; European Union. © Munksgaard, 1999.

Accepted for publication 14 October 1998

Regulatory Classification Work Regarding Skin Sensitizers

Regulatory classification of substances in the European Union (EU) is intended to identify their hazardous toxicological properties in a formal and harmonized manner. Such classification then communicates (in a standardized fashion across the whole of the EU) the need for appropriate risk assessment and risk management practices to be implemented for that substance.

The prevalence of contact allergy in the general population in Europe is about 10%. Of those sensitized, about 2–4% have ongoing allergic contact

dermatitis, which is a consequence of exposure to environmental chemicals. In clinical practice, no evidence of heredity to contact allergy is observed, although there seems to be a genetic predisposition to the disease (1). Exposure factors appear to be of much greater importance than genetic predisposition. Limitation of exposure to sensitizing substances is therefore of the utmost importance in the prevention of allergic contact dermatitis. This primary prevention is focused on avoiding the induction of skin sensitization. Every year, thousands of new chemicals are introduced on the world market. QSAR studies and predictive tests in animals are used to try to identify those new

substances with contact allergenic properties. However, from clinical practice we learn of new substances with allergenic properties that are still introduced on the market in addition to those already there. In preventive work, it is therefore important to classify and label substances and materials with regard to their sensitizing capacity, so as to limit their exposure in workplaces and in daily life.

According to EU legislation (2), substances and preparations shall be classified as sensitizing and assigned the risk phrase R43: "May cause sensitization by skin contact" if practical experience shows the substance or preparation to be capable of inducing sensitization by skin contact in a substantial number of persons or when positive results from an appropriate animal test are given. Comments regarding the use of R43 as given by the authorities are reproduced in Table 1. It is a long process before the decision is taken as to whether or not a substance shall be formally classified and labelled. First, a proposal from an EU country that a certain substance should be classified as a skin sensitizer is put forward. The proposal is discussed in the Commission Working Group on the classification and labelling of dangerous sub-

stances. The work of this group is directly associated with the Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. The work within the group is lead by the European Chemical Bureau associated to Directorate General (DG) XI, which handles environment, nuclear safety and civil protection. Decisions taken by the working group are in the form of advice given to the Committee for Adaption to Technical Progress (ATP). In the yearly ATP meetings of this committee, the EU member countries vote for or against the proposed changes in different appendices to the Council Directive 67/548/EEC.

Annex 1 (3) contains the list of classified substances and preparations. The formal decision is taken by the European Parliament.

WHO (4) has recently proposed criteria for the classification of skin and airway sensitizing substances. In the proposed criteria, it is suggested that the most important method to identify contact allergens is clinical evaluation and patch testing, i.e., human evidence. Data from more than 1 patient and from more than 1 independent centre are required. Furthermore the results from adequate predictive tests in at least 2 separate animal studies, of which 1 must be in guinea pigs, are considered as sufficient evidence for contact sensitizing capacity of a substance. The exposed animals must show a statistically significant reactivity compared to non-sensitized sham-treated control animals. Recommendations for prevention have been presented (5). In the OECD guidelines (6), 2 guinea pig methods are recommended. 1 method is the guinea pig maximization test (GPMT) (7, 8), with both intradermal and epicutaneous exposure of the compound and the use of Freund's complete adjuvant (FCA) to increase the immune response. The other is the Buehler method with only epicutaneous exposure and no FCA treatment (9–11). Whilst these methods may identify intrinsic skin sensitizing hazards, to make a risk assessment the following criteria also have to be taken into consideration: assessment of dose versus response, measurement or estimation of the intensity, frequency and duration of exposure.

In regulatory work, a specific chemical with its intrinsic molecular structure is classified as a skin sensitizer. This implies that the compound is stable throughout its lifetime. However, in reality, compounds may change when used in production, when stored or when handled by the consumer. Recently, the awareness of the instability of specific compounds has increased. Thus, there have been discussions about how to classify such compounds within the Commission Working Group on the

Table 1. Comments on the evidence that is sufficient to classify a substance with R43; reproduced from the Official J of the European Communities

Human evidence

Without supportive evidence

- Positive data from appropriate patch testing, normally in more than one dermatological clinic, or
- Epidemiological studies showing allergic contact dermatitis caused by the substance. Situations in which a high proportion of those exposed exhibit characteristic symptoms are to be looked at with special concern, even if the number of cases is small, or
- Positive data from experimental studies in man.

Supportive evidence is necessary

- Isolated episodes of allergic contact dermatitis, or
- Epidemiological studies where chance, bias or confounders have not been ruled out fully with reasonable confidence.

Examples of supportive evidence

- Data from animal tests performed according to existing guidelines, with a result that does not meet the criteria given in the section on animal studies but is sufficiently close to the limit to be considered significant, or
- Data from non-standard methods, or
- Appropriate structure-activity relationships.

Animal studies

- In the case of the adjuvant type test method* for skin sensitisation or in the case of other adjuvant type test methods, a response of at least 30% of the animals is considered as positive. For any other test method** a response of at least 15% of the animals is considered positive.

* Guinea pig maximization test is the official method (7, 8).

** Buehler test is the official method (9–11).

classification and labelling of dangerous substances.

The purpose of the present work is to discuss the problem of skin sensitizing oxidation/degradation products formed by air exposure of various materials or substances with very low allergenic activity. Should we classify a substance as allergenic due to its inherent ability to form allergenic oxidation products during normal storage and/or use? Or should the classification be based on the substance itself as seen in the original structure? It is the aim of this work to suggest guidelines on how to handle this problem from the point of view of regulatory classification.

Classification of Limonene

About a decade ago, *d*-limonene, the main compound in citrus oil, was introduced as an environmentally friendly alternative to the organic solvents commonly employed for defatting and cleaning in industry, and also used for hand-cleansers. However, the toxicological effects of *d*-limonene were not well documented, and experimental sensitization studies showed diverging results (12). Recent investigations (13–15) show that *d*-limonene itself has a very low sensitizing capacity, but that oxidation products of *d*-limonene are formed on exposure to the air. The majority of the oxidation products identified were found to be contact allergens (Fig. 1). From a mechanistic point of view, the change in allergenic activity when limonene is oxidized can be explained by the change in molecular reactivity. To become a sensitizer, a molecule (hapten) has to bind to macromolecules, e.g., proteins in the skin, to form an antigen. The proteins contain reactive nucleophilic (electron rich) sites, such as amino- and thiol-groups, that can bind to electrophilic (electron deficient) sites in other molecules to form a covalent bond. An oxygen atom introduced into the limonene molecule attracts electrons, which makes a carbon atom in the molecule electron deficient. Thus an electrophilic site is created in the molecule, which is susceptible to nucleophilic attack from electron-rich sites in protein (16). Carvone and limonene oxide are examples of molecules with electrophilic sites (Fig. 1). Antigen formation can also occur via a radical mechanism. This is most probably the case with the limonene hydroperoxides, which are strong contact allergens (15, 17, 18). Thus, various haptens are formed from *d*-limonene during air exposure, which in turn form different antigens not capable of cross-reacting. In this way, patients reacting to oxidized *d*-limonene can have different allergies. Testing with isolated

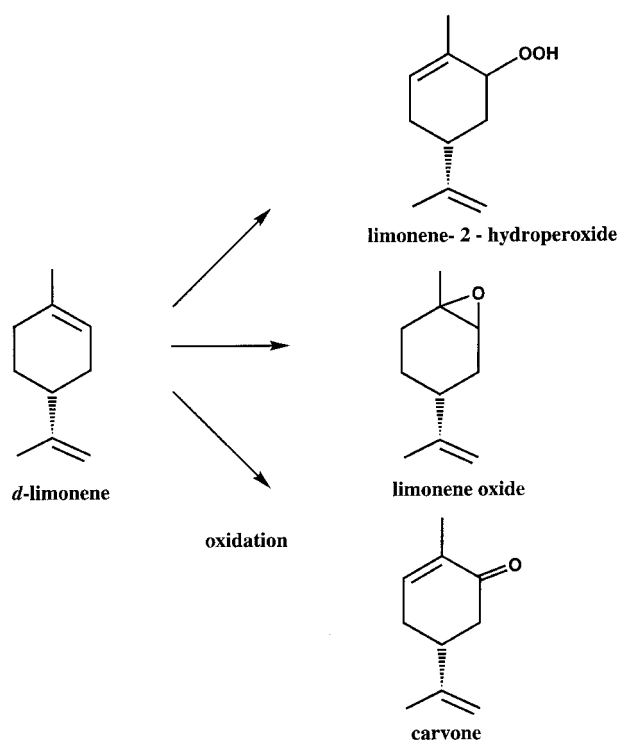


Fig. 1. *d*-limonene and skin sensitizing oxidation products obtained on air exposure.

haptens will not detect all cases of contact allergy caused by oxidized *d*-limonene (19).

Within EU regulatory work, it was decided by the Commission Working Group on the classification and labelling of dangerous substances (lead by the European Chemicals Bureau under DG XI) that *d*-limonene, as well as its enantiomer *l*-limonene and the racemic form dipentene, should be classified as skin sensitizers at the ATP meeting in 1998. The effective basis for the classification is the formation of skin sensitizers consequent upon air exposure of limonene. Addition of antioxidants such as butylated hydroxytoluene (BHT) will prevent the formation of oxidation products, for as long as the antioxidant is present (20). If the product is consumed when the antioxidant is still present, it could be questioned why the product should be labelled with R43 since *d*-limonene itself is not allergenic. Classification of *d*-limonene as a skin sensitizer will also lead to difficulties for structure-activity analysis, since there is a dissociation between structure and toxicity.

Classification of Colophony

The debate regarding the classification of colophony (rosin) has been much more vigorous compared to that of limonene, since the industrial interest in colophony is much more pronounced.

About 1 million tons of colophony are produced annually in the world. It has widespread use and is present in a great variety of products used at work, in consumer products and in leisure activities. It is one of the most common causes of positive patch test reactions among dermatitis patients when they are screened for contact allergy (21). Colophony tapped from living pine trees is named gum rosin, colophony from pine stumps wood rosin, and colophony obtained as a by-product in the pulp industry tall oil rosin. It has a complex chemical composition, consisting of 90% diterpenoid resin acids and 10% neutral material. The main resin acids are abietic acid and dehydroabietic acid. The abietic type acids (Fig. 2) are easily oxidized when in contact with air, and the oxidation products have been identified as contact sensitizers (22–25). Oxidation gives increased reactivity regarding the sensitizing effect, as was discussed for limonene (26, 27). Like limonene, colophony is part of the antimicrobial defence system of its host. When a tree is damaged and the exudate, containing diterpenoids, comes into contact with air, the molecules are activated by oxidation and acquire antimicrobial properties. Thus, such properties are inherent.

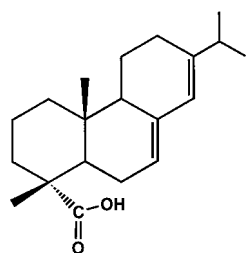
In 1993, colophony was classified as both a skin sensitizer and a sensitizer by inhalation at the ATP meeting. In 1994, the ATP meeting retained only the classification regarding skin sensitization. All types of colophony (gum rosin, wood rosin and tall oil rosin; CAS no 8050-09-7, 8052-10-6, 73138-82-6) were included in the classification. The Commission, member states and representatives of the industry considered that identification of sensitizing colophony and colophony derivatives was very complex and declared that all such related substances listed in European Inventory of New and Existing Chemical Substances (EINECS) would be considered as to their sensitizing properties, on the basis of the information which the industrial sector concerned would provide before the beginning of 1996. The colophony industry started to test the

allergenic activity of different qualities of colophony. They confirmed that colophony directly obtained and kept under nitrogen had a very low allergenic activity, while air-exposed colophony had a sensitizing effect (personal communication, A-T Karlberg). It was then debated whether colophony should be classified, since nascent colophony shows no significant sensitizing potency, in contrast to colophony after contact with air, which has a significant skin sensitizing capacity. However, unlike limonene, the chemical composition of colophony is very complex. Therefore, it is not possible to make a definitive identification of the allergenic and non-allergenic material. The oxidation process starts immediately and not all allergenic oxidation products have been identified. So far, it has not been possible to define a specific analytical method(s) to identify and quantify all the contact allergenic oxidation products in the material. The existing 1994 classification of colophony within the EU still stands but it continues to be under debate.

Discussion

Classification of substances in terms of their ability to behave as skin sensitizers is quite a complex matter. Whilst it is of course easy to classify potent allergens such as dichloronitrobenzene and chloromethylisothiazolinone, other chemicals may be rather weak allergens and the evidence that they do sensitize to any significant degree correspondingly inconclusive (e.g., octanol). Other materials, such as isoeugenol and p-phenylenediamine (PPD), are prohaptens, which are not chemically reactive agents but are rendered as such by metabolism in the skin (29, 30). What all these substances have in common is that skin contact with the specific chemical can give rise to contact allergy. In contrast, the types of materials that we are discussing are not themselves contact allergens, but must be oxidized in air to yield reactive species which then cause sensitization.

For the 1st group of substances, it is quite reasonable that, if the evidence is sufficient, they should be formally classified according to the EU scheme and labelled with R43. In the 2nd group, each substance should be considered individually. Where air oxidation yields significantly sensitizing species, classification is appropriate unless adequate antioxidant measures can be put in place. However, if a positive classification is made, then a note (e.g., in Annex I) should indicate that the primary chemical structure of the notified substance is not a skin sensitizer, but that (some of) its oxidation products are. Thus, for example, substances such as limonene and abietic acid should only be classified (on the basis of sufficient evi-



abietic acid

Fig. 2. The major component in colophony, abietic acid, is easily oxidized on air exposure.

dence) as R43 with an appropriate note indicating that it is oxidation species that are responsible. On the other hand, with naturally occurring complex mixtures, such as colophony, which inevitably contain sensitizing oxidation products, these should then (on the basis of sufficient evidence) be classified with R43.

We have given examples on natural terpenoid materials, but the concept of allergens formed by air oxidation can apply to other materials widely used in industrial products. What should be considered is the intrinsic susceptibility of a chemical to air oxidation (autoxidation), rather than its origin.

Classification discriminating (based on scientific data) between the pure compound and the compound together with its allergenic oxidation products will give manufacturers the opportunity to improve their products with regard to production, handling and storage. The formation of contact allergens on air exposure might be avoided by production under nitrogen. The storage time could be reduced and storage at low temperatures be recommended when possible. Antioxidants can in some cases offer a solution to the problem. However, the use of antioxidants is not free from problems. The effect of the antioxidant butylated hydroxytoluene (BHT) on the formation of oxidation products in air-exposed *d*-limonene has been studied (20). No oxidation was seen when BHT was present, but oxidation started as soon as BHT became consumed. The length of time before onset of oxidation depends on the purity of the limonene and on its storage temperature, and has to be carefully calculated. Furthermore, antioxidants themselves may cause allergic contact dermatitis (31). The sensitizing risk of BHT was evaluated in a study based both on consecutively patch tested patients and on estimates of exposure derived from a national database on chemical products (32). No positive patch test reactions were found to BHT 2% pet. in 1336 consecutively tested dermatitis patients, although BHT is frequently used in both industrial and consumer products in concentrations below 1%. The conclusion drawn from this study was that the concentrations of BHT normally used could be regarded as safe in relation to contact dermatitis.

Conclusions and Recommendations

In regulatory classification work on skin sensitizers, the intrinsic susceptibility of a chemical to air oxidation should be taken into consideration. We have given examples of natural terpenoid materials, but the concept of allergens formed by air

oxidation can apply to other materials widely used in industrial products.

If a positive classification is made for a substance with a known chemical structure, a note should indicate that the primary chemical structure of the notified substance is not a skin sensitizer, but that (some of) its oxidation products are.

Complex mixtures which inevitably contain sensitizing oxidation products should (on the basis of sufficient evidence) be classified with R43.

References

1. Menné T, Holm N V. Nickel allergy in a female twin population. *Int J Dermatol* 1983; 22: 22–28.
2. European Communities. (1993) Annex IV to Commission Directive 93/2 1/EEC of 27 April 1993 adapting to technical progress for the 18th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Official Journal of the European Communities* L110A 36: 59.
3. EC (1996) Annex to Commission Directive 96/54/EC. *Official Journal of the European Communities* L 248: 1–230 EC Annex 1.
4. World Health Organisation. Criteria for classification of the skin and airway sensitizing substances in the work and general environments Flyvholm M-A, Andersen K E, Baranski B, Sarlo K (eds). WHO, 1997.
5. Vos J G, Younes M, Smith E. (eds) *Allergic hypersensitivities induced by chemicals. Recommendations for prevention*. Boca Raton: CRC Press, 1996. Published on behalf of the World Health Organization Regional Office for Europe.
6. OECD (1992) Organisation for Economic Cooperation and Development: *Guidelines for the testing of chemicals: 406: Skin sensitisation*. Adopted 17 July 1992.
7. Magnuson B, Kligman A M. *Allergic contact dermatitis in the guinea pig*. Springfield: Thomas; 1970.
8. Wahlberg J E, Boman A. Guinea pig maximization test. In: Andersen K, Maibach H I (eds). *Curr Probl Derm* 1985; 14: 59–106. Basel: Karger.
9. Buehler, E V. Delayed contact hypersensitivity in the guinea pig. *Arch Derm* 1965; 91: 171–177.
10. Ritz H L, Buehler, E V. Planning, conduct and interpretation of guinea pig sensitization patch tests. In: Drill V A, Lazar P (eds): *Current concepts in cutaneous toxicity*. New York: Academic Press, 1980: 25–40.
11. Buchler, E V. A rationale for the selection of occlusion to induce and elicit delayed contact hypersensitivity in the guinea pig. A prospective test. In: Andersen K, Maibach H I (eds) *Curr Probl Derm* 1985; 14: 39–58. Basel: Karger.
12. Karlberg A-T, Lindell B. Limonene. In: Criteria Documents from the Nordic Expert Group 1993. Nordic Councils of Ministers. Beijer B, Lundberg P, (eds): *Arbete & Häl-sa* 1993; 35: 207–246.
13. Karlberg A-T, Boman A, Melin B. Animal experiments on the allergenicity of *d*-limonene – the citrus solvent. *Ann Occup Hyg* 1991; 35: 419–426.
14. Karlberg A-T, Magnusson K, Nilsson U. Air oxidation of *d*-limonene – the citrus solvent-creates potent allergens. *Contact Dermatitis* 1992; 26: 332–340.
15. Karlberg A-T, Shao LP, Nilsson U, Gäfvert E, Nilsson J L G. Hydroperoxides in oxidized *d*-limonene identified as potent contact allergens. *Arch Derm Res* 1994; 286: 97–103.
16. Roberts D W, Lepoittevin J-P. Hapten – protein interactions. In: Lepoittevin J-P, Basketter D, Goossens A,

- Karlberg A-T (eds): *Allergic contact dermatitis. The molecular basis*. Berlin, Heidelberg, New York: Springer-Verlag, 1997: 81–112.
17. Lepoittevin J-P, Karlberg A-T. Interactions of allergenic hydroperoxides with proteins. A radical mechanism? *Chem Res Toxicol* 1994; 7: 130–133.
 18. Bézard M, Karlberg A-T, Montelius J, Lepoittevin J-P. Studies on the allergenic activity of monoterpene hydroperoxides and epoxides. *Chem Res Toxicol* 1997; 10: 987–993.
 19. Karlberg A-T, Doms-Gossens A. Contact allergy to oxidized *d*-limonene – the citrus solvent. *Contact Dermatitis* 1997; 36: 201–206.
 20. Karlberg A-T, Magnusson K, Nilsson U. Influence of an antioxidant on the formation of allergenic compounds during autooxidation of *d*-limonene. *Ann Occup Hyg* 1994; 38: 199–207.
 21. Andersen K E, Burrows D, White I R. Allergens from the standard series. In: Rycroft R J G, Menné T, Frosch P J (eds): *Textbook of contact dermatitis*, 2nd edition. Berlin, Heidelberg, New York: Springer-Verlag, 1995: 429–430.
 22. Karlberg A-T, Bohlinder K, Boman A, Hacksell U, Hermansson J, Jacobsson S, Nilsson J L G. Identification of 15-hydroperoxyabietic acid as a contact allergen in Portuguese colophony. *J Pharm Pharmacol* 1988; 40: 42–47.
 23. Karlberg A-T, Boman A, Hacksell U, Jacobsson S, Nilsson J L G. Contact allergy to dehydroabietic acid derivatives isolated from Portuguese colophony. *Contact Dermatitis* 1988; 19: 166–174.
 24. Gäfvert E, Shao L P, Karlberg A-T, Nilsson U, Nilsson J L G. Contact allergy to resin acid hydroperoxides. Hapten binding via free radicals and epoxides. *Chem Res Toxicol* 1994; 7: 260–266.
 25. Krohn K, Budantio E, Flörke U, Hausen B M. Untersuchungen der allergenen Prinzipien aus Kolophonium: Autoxidation, Synthese und Sensibilisierung. *Liebigs Ann Chem* 1992; 911–919.
 26. Karlberg A-T. Air oxidation increases the allergenic potential of tall oil rosin. Gum rosin contact allergens also identified in tall oil rosin. *Am J Contact Dermatitis* 1991; 2: 43–49.
 27. Hausen B M, Bories M, Budantio E, Krohn K. Contact allergy due to colophony. IX. Sensitization studies with further products isolated after oxidative degradation of resin acids and colophony. *Contact Dermatitis* 1993; 29: 234–240.
 29. Krasteva M, Nicolas J F, Chabeau G, Garrigue J L, Bour H, Thivolet J, Schmitt D. Dissociation of allergenic and immunogenic functions in contact sensitivity to paraphenylenediamine. *Int Arch Allergy Immunol* 1993; 102: 200–204.
 30. Bertrand F, Basketter D A, Roberts D W, Lepoittevin J-P. Skin sensitization to eugenol and isoeugenol in mice: possible metabolic pathways involving o-quinone and quinonemethide intermediates. *Chem Res Toxicol* 1997; 10: 335–343.
 31. De Groot A C, White I R. Cosmetics and skin care products. In: Rycroft R J G, Menné T, Frosch P J. (Eds) *Textbook of contact dermatitis*, 2nd edition. Berlin, Heidelberg, New York: Springer-Verlag, 1995: 470–471.
 32. Flyvholm M-A, Menné T. Sensitizing risk of butylated hydroxytoluene based on exposure and effect data. *Contact Dermatitis* 1990; 23: 341–345.

Address:

Ann-Therese Karlberg
Department of Occupational Medicine
Dermatology Division
National Institute for Working Life
S-171 84 Solna
Sweden