**Committee of Experts on the Transport of Dangerous Goods and on the Globally Harmonized System of Classification and Labelling of Chemicals**

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**Work on the Globally Harmonized System of Classification and Labelling of Chemicals: use of non-animal testing methods for classification of health hazards**

# Revision of Chapter 3.4 to fully incorporate non-animal testing methods for skin sensitization

## Transmitted by the experts from the United Kingdom and the Netherlands on behalf of the Informal Working Group on the use of non-animal testing methods for classification of health and environmental hazards

This informal document sets out the changes proposed in document ST/SG/AC.10/C.4/2022/14 as amended by informal document UN/SCEGHS/43/INF.8. Existing (unchanged) text is shown in black, with new text (from that proposed in ST/SG/AC.10/C.4/2022/14 and INF.3) shown in red. Deleted text (from that proposed in ST/SG/AC.10/C.4/2022/14 and INF.3) is shown in blue ~~strikethrough~~ text. Text shown in plain blue is the text previously proposed in ST/SG/AC.10/C.4/2022/14 and INF.3 that remains unchanged.

# “CHAPTER 3.4

# RESPIRATORY OR SKIN SENSITIZATION

## 3.4.1 Definitions and general considerations

3.4.1.1 *Respiratory sensitization* refers to hypersensitivity of the airways occurring after inhalation of a substance or a mixture.

*Skin sensitization* refers to an allergic response occurring after skin contact with a substance or a mixture.

3.4.1.2 For the purpose of this chapter, sensitization includes two phases: the first phase is induction of specialized immunological memory in an individual by exposure to an allergen. The second phase is elicitation, i.e. production of a cell-mediated or antibody-mediated allergic response by exposure of a sensitized individual to an allergen.

3.4.1.3 For respiratory sensitization, the pattern of induction followed by elicitation phases is shared in common with skin sensitization. For skin sensitization, an induction phase is required in which the immune system learns to react; clinical symptoms can then arise when subsequent exposure is sufficient to elicit a visible skin reaction (elicitation phase). As a consequence, predictive tests usually follow this pattern in which there is an induction phase, the response to which is measured by a standardized elicitation phase, typically involving a patch test. The local lymph node assay is the exception, directly measuring the induction response. Evidence of skin sensitization in humans normally is assessed by a diagnostic patch test.

3.4.1.4 Usually, for both skin and respiratory sensitization, lower levels are necessary for elicitation than are required for induction. Provisions for alerting sensitized individuals to the presence of a particular sensitizer in a mixture can be found in 3.4.4.2.

3.4.1.5 The hazard class “respiratory or skin sensitization” is differentiated into:

(a) Respiratory sensitization; and

(b) Skin sensitization

## 3.4.2 Classification criteria for substances

### 3.4.2.1 *Respiratory sensitizers*

3.4.2.1.1 *Hazard categories*

3.4.2.1.1.1 Respiratory sensitizers shall be classified in Category 1 where sub-categorization is not required by a competent authority or where data are not sufficient for sub-categorization.

3.4.2.1.1.2 Where data are sufficient and where required by a competent authority, a refined evaluation according to 3.4.2.1.1.3 allows the allocation of respiratory sensitizers into sub-category 1A, strong sensitizers, or sub-category 1B for other respiratory sensitizers.

3.4.2.1.1.3 Effects seen in either humans or animals will normally justify classification in a weight of evidence approach for respiratory sensitizers. Substances may be allocated to one of the two sub-categories 1A or 1B using a weight of evidence approach in accordance with the criteria given in Table 3.4.1 and on the basis of reliable and good quality evidence from human cases or epidemiological studies and/or observations from appropriate studies in experimental animals.

**Table 3.4.1: Hazard category and sub-categories for respiratory sensitizers**

| **CATEGORY 1:** | **Respiratory sensitizer** |
|---|---|
| | A substance is classified as a respiratory sensitizer:<br>(a) if there is evidence in humans that the substance can lead to specific respiratory hypersensitivity and/or<br>(b) if there are positive results from an appropriate animal test[1]. |
| **Sub-category 1A:** | Substances showing a high frequency of occurrence in humans; or a probability of occurrence of a high sensitization rate in humans based on animal or other tests[1]. Severity of reaction may also be considered. |
| **Sub-category 1B:** | **Substances showing a low to moderate frequency of occurrence in humans; or a probability of occurrence of a low to moderate sensitization rate in humans based on animal or other tests[1]. Severity of reaction may also be considered.** |

3.4.2.1.2 *Human evidence*

3.4.2.1.2.1 Evidence that a substance can lead to specific respiratory hypersensitivity will normally be based on human experience. In this context, hypersensitivity is normally seen as asthma, but other hypersensitivity reactions such as rhinitis/conjunctivitis and alveolitis are also considered. The condition will have the clinical character of an allergic reaction. However, immunological mechanisms do not have to be demonstrated.

3.4.2.1.2.2 When considering the human evidence, it is necessary for a decision on classification to take into account, in addition to the evidence from the cases:

(a) the size of the population exposed;

(b) the extent of exposure.

3.4.2.1.2.3 The evidence referred to above could be:

(a) clinical history and data from appropriate lung function tests related to exposure to the substance, confirmed by other supportive evidence which may include:

(i) *in vivo* immunological test (e.g. skin prick test);

(ii) *in vitro* immunological test (e.g. serological analysis);

(iii) studies that may indicate other specific hypersensitivity reactions where immunological mechanisms of action have not been proven, e.g. repeated low-level irritation, pharmacologically mediated effects;

(iv) a chemical structure related to substances known to cause respiratory hypersensitivity;

(b) data from positive bronchial challenge tests with the substance conducted according to accepted guidelines for the determination of a specific hypersensitivity reaction.

3.4.2.1.2.4 Clinical history should include both medical and occupational history to determine a relationship between exposure to a specific substance and development of respiratory hypersensitivity. Relevant information includes aggravating factors both in the home and workplace, the onset and progress of the disease, family history

[1] *At present, recognized and validated animal models for the testing of respiratory hypersensitivity are not available. Under certain circumstances, data from animal studies may provide valuable information in a weight of evidence assessment.*

and medical history of the patient in question. The medical history should also include a note of other allergic or airway disorders from childhood, and smoking history.

3.4.2.1.2.5 The results of positive bronchial challenge tests are considered to provide sufficient evidence for classification on their own. It is however recognized that in practice many of the examinations listed above will already have been carried out.

3.4.2.1.3 *Animal studies*

Data from appropriate animal studies[1] which may be indicative of the potential of a substance to cause sensitization by inhalation in humans[2] may include:

(a) measurements of Immunoglobulin E (IgE) and other specific immunological parameters, for example in mice;

(b) specific pulmonary responses in guinea pigs.

**3.4.2.2 *Skin sensitizers***

3.4.2.2.1 *Hazard categories*

3.4.2.2.1.1 Skin sensitizers shall be classified in Category 1 where sub-categorization is not required by a competent authority or where data are not sufficient for sub-categorization.

3.4.2.2.1.2 Where data are sufficient and where required by a competent authority, a refined evaluation according to 3.4.2.2.2 – 3.4.2.2.6 allows the allocation of skin sensitizers into sub-category 1A, strong sensitizers, or sub-category 1B for other skin sensitizers.

3.4.2.2.1.3 For classification of skin sensitizers, all available and relevant information is collected and its quality in terms of adequacy and reliability is assessed. Classification should be based on mutually acceptable data/results generated using methods and/or defined approaches that are validated according to international procedures. These include both OECD guidelines and equivalent methods/defined approaches (see 1.3.2.4.3). Sections 3.4.2.2.2 to 3.4.2.2.6 provide classification criteria for the different types of information that may be available.

3.4.2.2.1.4 A tiered approach (see 3.4.2.2.7) organizes the available information on skin sensitization into levels/tiers and provides for decision-making in a structured and sequential manner. Classification results directly when the information consistently satisfies the criteria. However, where the available information gives inconsistent and/or conflicting results within a tier, classification of a substance is made on the basis of the weight of evidence within that tier. In some cases when information from different tiers gives inconsistent and/or conflicting results (see 3.4.2.2.7.7) or where data individually are insufficient to conclude on the classification, an overall weight of evidence assessment is used (see 1.3.2.4.9 and 3.4.2.2.7.6).

3.4.2.2.1.5 Guidance on the interpretation of criteria and references to relevant guidance documents are provided in 3.4.5.3.

3.4.2.2.2 *Classification based on human data (Tier 1 in Figure 3.4.1)*

3.4.2.2.2.1 A substance is classified as a skin sensitizer in category 1 if there is evidence in humans that the substance can lead to sensitization by skin contact in a substantial number of persons.

[1] *At present, recognized and validated animal models for the testing of respiratory hypersensitivity are not available. Under certain circumstances, data from animal studies may provide valuable information in a weight of evidence assessment.*

3.4.2.2.2 Substances showing a high frequency of occurrence in humans, can be presumed to have the potential to produce significant sensitization and are classified in category 1A. Severity of reaction may also be considered. Human evidence for sub-category 1A can include:

(a) positive responses at ≤ 500 µg/cm[2] (Human Repeated Insult Patch Test (HRIPT), Human maximization test (HMT) – induction threshold);

(b) diagnostic patch test data where there is a relatively high and substantial incidence of reactions in a defined population in relation to relatively low exposure;

(c) other epidemiological evidence where there is a relatively high and substantial incidence of allergic contact dermatitis in relation to relatively low exposure.

3.4.2.2.3 Substances showing a low to moderate frequency of occurrence in humans can be presumed to have the potential to produce sensitization and are classified in category 1B. Severity of reaction may also be considered. Human evidence for sub-category 1B can include:

(a) positive responses at > 500 µg/cm[2] (HRIPT, HMT – induction threshold);

(b) diagnostic patch test data where there is a relatively low but substantial incidence of reactions in a defined population in relation to relatively high exposure;

(c) other epidemiological evidence where there is a relatively low but substantial incidence of allergic contact dermatitis in relation to relatively high exposure.

3.4.2.2.3 *Classification based on standard animal data (Tier 1 in Figure 3.4.1)*

3.4.2.2.3.1 A substance is classified as a skin sensitizer if there are positive results from an appropriate animal test. For Category 1, when an adjuvant type test method for skin sensitization is used, a response of at least 30 % of the animals is considered as positive. For a non-adjuvant Guinea pig test method a response of at least 15 % of the animals is considered positive. For Category 1, a stimulation index of three or more is considered a positive response in the local lymph node assay. Test methods for skin sensitization are described in the OECD Guideline 406 (the Guinea Pig Maximisation test and the Buehler guinea pig test) and Guideline 429 (Local Lymph Node Assay). Other methods may be used provided that they are well-validated and scientific justification is given. The Mouse Ear Swelling Test (MEST), appears to be a reliable screening test to detect moderate to strong sensitizers, and can be used as a first stage in the assessment of skin sensitization potential.

3.4.2.2.3.2 Substances showing a high potency in animals, can be presumed to have the potential to produce significant sensitization in humans and are classified in category 1A. Severity of reactions may also be considered. Animal test results for sub-category 1A can include data with values indicated in Table 3.4.2 below:

**Table 3.4.2: Animal test results for sub-category 1A**

| Assay | Criteria |
|---|---|
| Local lymph node assay | EC3 value ≤ 2 % |
| Guinea pig maximisation test | ≥ 30 % responding at ≤ 0.1 % intradermal induction dose or<br>≥ 60 % responding at > 0.1 % to ≤ 1 % intradermal induction dose |
| Buehler assay | ≥15 % responding at ≤ 0.2 % topical induction dose or<br>≥ 60 % responding at > 0.2 % to ≤ 20 % topical induction dose |

[2] *The mechanisms by which substances induce symptoms of asthma are not yet fully known. For preventative measures, these substances are considered respiratory sensitizers. However, if on the basis of the evidence, it can be demonstrated that these substances induce symptoms of asthma by irritation only in people with bronchial hyperreactivity, they should not be considered as respiratory sensitizers.*

3.4.2.2.3.3 Substances showing a low to moderate potency in animals can be presumed to have the potential to produce sensitization in humans and are classified in category 1B. Severity of reaction may also be considered. Animal test results for sub-category 1B can include data with values indicated in Table 3.4.3 below:

**Table 3.4.3: Animal test results for sub-category 1B**

| Assay | Criteria |
|---|---|
| Local lymph node assay | EC3 value > 2 % |
| Guinea pig maximisation test | ≥ 30 % to < 60 % responding at > 0.1 % to ≤ 1 % intradermal induction dose or<br>≥ 30 % responding at > 1 % intradermal induction dose |
| Buehler assay | ≥ 15 % to < 60 % responding at > 0.2 % to ≤ 20 % topical induction dose or<br>≥ 15 % responding at > 20 % topical induction dose |

3.4.2.2.4 *Classification based on defined approaches (Tier 1 or Tier 2 in Figure 3.4.1)*

3.4.2.2.4.1 Defined approaches consist of a rule-based combination of data obtained from a predefined set of different information sources (e.g. *in chemico* methods, *in vitro* methods, physico-chemical properties, non-test methods). It is recognized that most single non animal methods are not able to replace *in vivo* methods fully for most regulatory endpoints. Thus, defined approaches can be useful strategies of combining data for classifying substances and mixtures. Results obtained with a defined approach validated according to international procedures, such as OECD Guideline 497 or an equivalent approach, are conclusive for classification for skin sensitization if the criteria of the defined approach are fulfilled (see Table 3.4.6)[3]. Data from a defined approach can only be used for classification when the tested substance is within the applicability domain of the defined approach used. Additional limitations described in the published literature should also be taken into consideration.

3.4.2.2.4.2 Where the results from defined approaches are assigned a level of confidence as for example in OECD Guideline 497, a low confidence outcome of a defined approach in Tier 1 is inconclusive and thus cannot be used on its own to classify but may be considered in combination with other data in Tier 2.

[3.4.2.2.4.3 ~~Some evidence can be used individually and in defined approaches.~~ Individual evidence ~~used~~ considered within a defined approach~~es~~ should ~~then~~ not ~~also~~ be ~~used individually~~ considered as an additional line of evidence within a weight of evidence assessment.]

3.4.2.2.5 *Classification based on in chemico/in vitro data (Tier 1 or Tier 2 in Figure 3.4.1)*

3.4.2.2.5.1 The currently available *in chemico/in vitro* methods address specific biological mechanisms leading to the acquisition of skin sensitization as described, for example, in the OECD Adverse Outcome Pathway for Skin Sensitisation (see OECD~~,~~ (2014)). Individual test methods that are validated according to international procedures and are accepted as stand-alone methods, can be used to conclude on the classification in Tier 1. A competent authority may decide whether to use the method described in Appendix III to OECD Test Guideline 442C as a stand-alone method to discriminate between category 1A and those not categorized as category 1A (see 3.4.5.3.5).

3.4.2.2.5.2 Other non stand-alone *in chemico/in vitro* methods that are validated according to international procedures such as OECD Test Guidelines 442C (Annex I and II), 442D and 442E, are accepted as supportive evidence and should within Tier 1 only be used in combination with other types of data in defined approaches. The use of these methods in Tier 2 is described in 3.4.2.2.7.5.

[3] *Additional defined approaches have been proposed for skin sensitization (OECD ~~2016b~~(2017)) but no classification criteria have yet been agreed internationally.*

3.4.2.2.5.3 Other validated *in chemico/in vitro* test methods accepted by some competent authorities are described in ~~3.4.5.3.6.1~~ 3.4.5.3.6.2 [4]. A competent authority may decide which classification criteria, if any, should be applied for these test methods to conclude on classification.

3.4.2.2.5.4 *In chemico/in vitro* data can only be used for classification when the tested substance is within the applicability domain of the test method(s) used. Additional limitations described in the published literature should also be taken into consideration.

3.4.2.2.6 *Classification based on non-test methods (Tier 2 in Figure 3.4.1)*

3.4.2.2.6.1 Classification, including the conclusion not classified, can be based on non-test methods, with due consideration of reliability and applicability, on a case-by-case basis. Non-test methods include computer models predicting qualitative structure activity relationships (structural alerts, SAR) or quantitative structure-activity relationships (QSARs), computer expert systems, and read-across using analogue and category approaches.

3.4.2.2.6.2 Read-across using analogue or category approaches requires sufficiently reliable test data on similar substance(s) and justification of the similarity of the tested substance(s) with the substance(s) to be classified. Where adequate justification of the read-across approach is provided, it has in general higher weight than (Q)SARs.

3.4.2.2.6.3 Classification based on (Q)SARs requires sufficient data and validation of the model. The validity of the computer models and the prediction should be assessed using internationally recognized principles for the validation of (Q)SARs. With respect to reliability, lack of alerts in a SAR or expert system is not sufficient evidence for no classification.

3.4.2.2.6.4 For conclusions on no classification from read-across and (Q)SARs the adequacy and robustness of the scientific reasoning and of the supporting evidence should be well substantiated and normally requires multiple negative substances with good structural and physical (related to toxicokinetics) similarity to the substance being classified, as well as a clear absence of positive substances with good structural and physical similarity to the substance being classified.

3.4.2.2.7 *Classification in a tiered approach (Figure 3.4.1)*

3.4.2.2.7.1 A tiered approach to the evaluation of information should be considered, where applicable (Figure 3.4.1), recognizing that not all tiers as well as information within a tier may be relevant. However, all available and relevant information of sufficient quality needs to be examined for consistency with respect to the resulting classification.

3.4.2.2.7.2 Tier 1 - Classification based on human data, standard animal data, defined approaches or stand-alone *in chemico/in vitro* methods

For classification of a substance, evidence in Tier 1 may include data from any or all of the following lines of evidence. Where information from data within Tier 1 is inconsistent and/or conflicting, the conclusion is determined in a weight of evidence assessment:

(a) Experimental studies in humans (e.g., predictive patch testing, HRIPT, HMT (see paragraph 1.3.2.4.7, criteria in 3.4.2.2.2.2 (a) and 3.4.2.2.2.3 (a) and guidance 3.4.5.3.2);

(b) Epidemiological studies (e.g., case control studies, prospective studies) assessing allergic contact dermatitis (see paragraph 1.3.2.4.7, criteria in 3.4.2.2.2.2 (b and c) and 3.4.2.2.2.3 (b and c) and guidance 3.4.5.3.2);

[4] *Additional in chemico/in vitro methods have been proposed for skin sensitization (see ~~3.4.5.3.6.1~~3.4.5.3.6.1) but no classification criteria have yet been agreed internationally.*

(c) Well-documented cases of allergic contact dermatitis (see criteria in 3.4.2.2.2.2 (b) and 3.4.2.2.2.3 (b) and guidance 3.4.5.3.2);

(d) Appropriate animal studies (see criteria in 3.4.2.2.3, and guidance 3.4.5.3.3);

(e) Defined approaches validated according to international procedures (see 3.4.2.2.4, guidance 3.4.5.3.4, and Table 3.4.6);

(f) Stand-alone *in chemico/in vitro* methods validated according to international procedures (see 3.4.2.2.5, guidance 3.4.5.3.5, and Table 3.4.7).

3.4.2.2.7.3 Tier 2 - Classification based on inconclusive data from Tier 1, non stand-alone *in chemico/in vitro* methods~~,~~ or non-test methods ~~or low confidence/inconclusive results from defined approaches~~

In case a definitive conclusion on classification, including sub-categorization where required by a competent authority, cannot be derived from Tier 1, additional lines of evidence shall be considered in a weight of evidence assessment in Tier 2. These may include:

(a) Data from non stand-alone *in chemico/in vitro* methods (see 3.4.2.2.5 and 3.4.5.3.5);

(b) Data from non-test methods (see 3.2.2.2.6).

3.4.2.2.7.4 Evidence from non stand-alone *in chemico/in vitro* methods and from non-test methods should not be considered at this stage if this data is already used in a defined approach under 3.4.2.2.7.2.

3.4.2.2.7.5 Individual non stand-alone *in chemico/in vitro* methods validated according to international procedures~~,~~ and non-test methods (including read-across) ~~and low confidence/inconclusive data from defined approaches~~ can be applied in a weight of evidence assessment together with inconclusive data from Tier 1 and should be used in this second Tier because they can usually not be used as stand-alone (with the exception of good quality read-across). However, a competent authority may decide that a positive result with one of these non stand-alone *in chemico/in vitro* methods, may be used on its own to classify in category 1 (see Table 3.4.7).

3.4.2.2.7.6 Tier 3 - Classification based on overall weight of evidence assessment, including additional indicators

In case a definitive conclusion on classification including sub-categorization where required by a competent authority, cannot be derived from the previous tiers, an overall weight of evidence assessment using expert judgment should be used that may include a combination of two or more indicators of skin sensitization as listed below.

(a) Isolated episodes of allergic contact dermatitis;

(b) Epidemiological studies of limited power, e.g. where chance, bias or confounders have not been ruled out fully with reasonable confidence;

(c) Data from animal tests, performed according to existing guidelines, which do not meet the criteria for a positive result described in 3.4.2.2.3, but which are sufficiently close to the limit to be considered significant;

(d) Data from non-standard methods;

3.4.2.2.7.7 Where information from the various tiers is inconsistent and/or conflicting with respect to the resulting classification, information of sufficient quality from a higher tier is generally given a higher weight than information from a lower tier. However, when information from a lower tier would result in a stricter classification than information from a higher tier and there is concern for misclassification, then classification is determined by an overall weight of evidence assessment (i.e. in Tier 3). For example, having consulted the guidance in 3.4.5.3 as appropriate, classifiers concerned with a negative result for skin sensitization in a Buehler study when there is

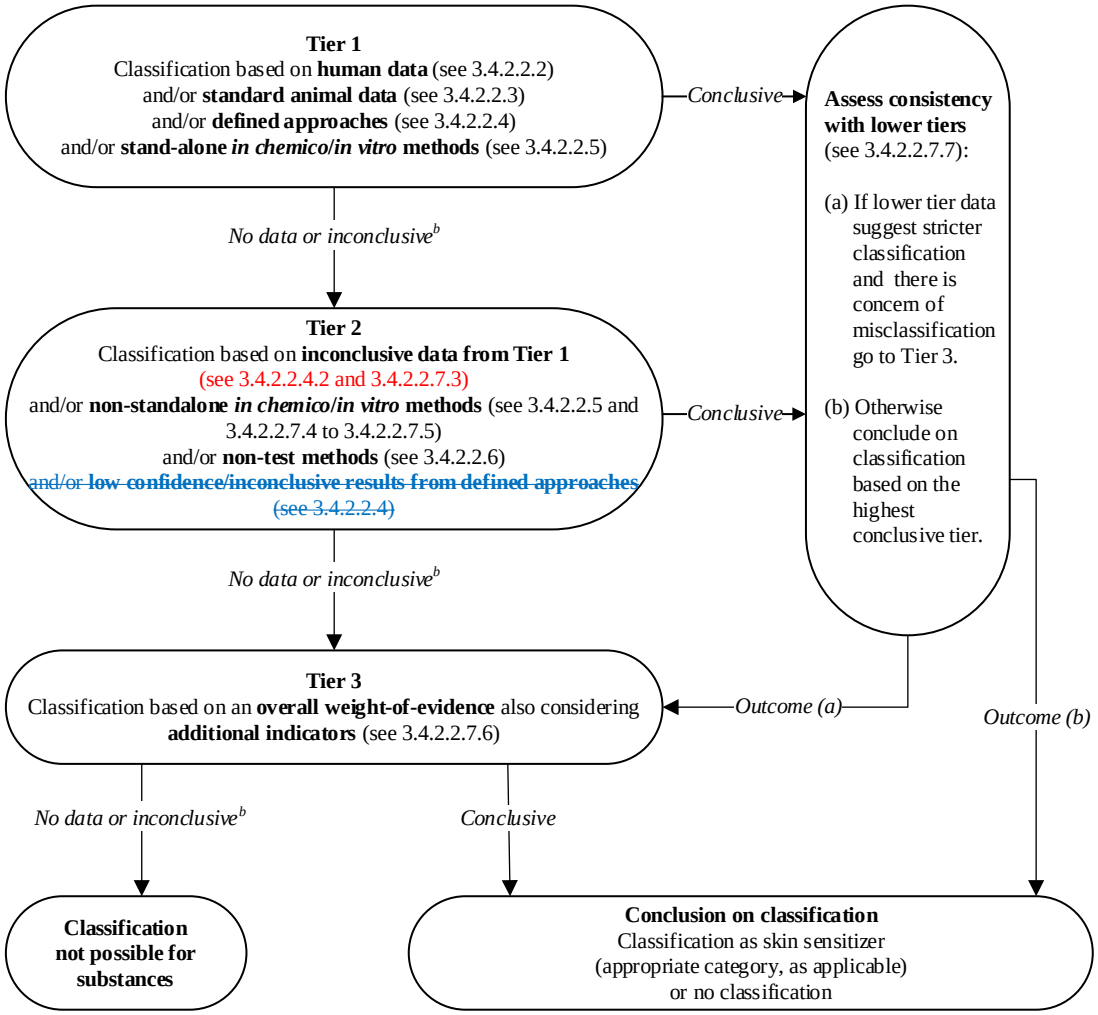
a clear positive result in humans for very similar substances (from read-across) would utilise an overall weight of evidence assessment.

3.4.2.2.8 Immunological contact urticaria

3.4.2.2.8.1 Substances meeting the criteria for classification as respiratory sensitizers may in addition cause immunological contact urticaria. Consideration should be given to classifying these substances also as skin sensitizers. Substances which cause immunological contact urticaria without meeting the criteria for respiratory sensitizers should also be considered for classification as skin sensitizers.

3.4.2.2.8.2 There is no recognized animal model available to identify substances which cause immunological contact urticaria. Therefore, classification will normally be based on human evidence which will be similar to that for skin sensitization.

**Figure 3.4.1: Application of the tiered approach for skin sensitization[a]**




[a] *Before applying the approach, the explanatory text in 3.4.2.2.7 as well as the guidance in 3.4.5.3 should be consulted. Only adequate and reliable data of sufficient quality should be included in applying the tiered approach.*

[b] *Information may be inconclusive for various reasons, e.g.:*

– *The available data may be of insufficient quality, or otherwise insufficient/inadequate for the purpose of classification, e.g. due to quality issues related to experimental design and/or reporting;*

– *Where competent authorities make use of the skin sensitization sub-categories 1A and 1B, the available data may not be capable of distinguishing between sub-category 1A and sub-category 1B.*

### 3.4.3 Classification criteria for mixtures

#### 3.4.3.1 *Classification of mixtures when data are available for the complete mixture*

When reliable and good quality evidence from human experience or appropriate studies in experimental animals, as described in the criteria for substances, is available for the mixture, then the mixture can be classified by weight of evidence evaluation of these data. Care should be exercised in evaluating data on mixtures that the dose used does not render the results inconclusive. (For special labelling required by some competent authorities, see the note to Table 3.4.4 of this chapter and 3.4.4.2.)

#### 3.4.3.2 *Classification of mixtures when data are not available for the complete mixture: bridging principles*

3.4.3.2.1 Where the mixture itself has not been tested to determine its sensitizing properties, but there are sufficient data on both the individual ingredients and similar tested mixtures to adequately characterize the hazards of the mixture, these data will be used in accordance with the following agreed bridging principles. This ensures that the classification process uses the available data to the greatest extent possible in characterizing the hazards of the mixture without the necessity for additional testing in animals.

3.4.3.2.2 *Dilution*

If a tested mixture is diluted with a diluent which is not a sensitizer and which is not expected to affect the sensitization of other ingredients, then the new diluted mixture may be classified as equivalent to the original tested mixture.

3.4.3.2.3 *Batching*

The sensitizing properties of a tested production batch of a mixture can be assumed to be substantially equivalent to that of another untested production batch of the same commercial product when produced by or under the control of the same manufacturer, unless there is reason to believe there is significant variation such that the sensitization potential of the untested batch has changed. If the latter occurs, a new classification is necessary.

3.4.3.2.4 *Concentration of mixtures of the highest sensitizing category/sub-category*

If a tested mixture is classified in Category 1 or sub-category 1A, and the concentration of the ingredients of the tested mixture that are in Category 1 and sub-category 1A is increased, the resulting untested mixture should be classified in Category 1 or sub-category 1A without additional testing.

3.4.3.2.5 *Interpolation within one category/sub-category*

For three mixtures (A, B and C) with identical ingredients, where mixtures A and B have been tested and are in the same category/sub-category, and where untested mixture C has the same toxicologically active ingredients as mixtures A and B but has concentrations of toxicologically active ingredients intermediate to the concentrations in mixtures A and B, then mixture C is assumed to be in the same category/sub-category as A and B.

3.4.3.2.6 *Substantially similar mixtures*

Given the following:

(a) Two mixtures: (i) A + B;
(ii) C + B;

(b) The concentration of ingredient B is essentially the same in both mixtures;

(c) The concentration of ingredient A in mixture (i) equals that of ingredient C in mixture (ii);

(d) Ingredient B is a sensitizer and ingredients A and C are not sensitizers;

(e) A and C are not expected to affect the sensitizing properties of B.

If mixture (i) or (ii) is already classified by testing, then the other mixture can be assigned the same hazard category.

3.4.3.2.7 *Aerosols*

An aerosol form of the mixture may be classified in the same hazard category as the tested non-aerosolized form of the mixture provided that the added propellant does not affect the sensitizing properties of the mixture upon spraying.

**3.4.3.3 *Classification of mixtures when data are available for all ingredients or only for some ingredients of the mixture***

The mixture should be classified as a respiratory or skin sensitizer when at least one ingredient has been classified as a respiratory or skin sensitizer and is present at or above the appropriate cut-off value/concentration limit for the specific endpoint as shown in Table 3.4.4 for solid/liquid and gas respectively.

**Table 3.4.4: Cut-off values/concentration limits of ingredients of a mixture classified as either respiratory sensitizers or skin sensitizers that would trigger classification of the mixture**

| **Ingredient classified as:** | **Cut-off values/concentration limits triggering classification of a mixture as:** | | |
|---|---|---|---|
| | **respiratory sensitizer Category 1** | | **skin sensitizer Category 1** |
| | **Solid/Liquid** | **Gas** | **All physical states** |
| **Respiratory sensitizer Category 1** | ≥ 0.1 % (see note) | ≥ 0.1 % (see note) | -- |
| | ≥ 1.0 % | ≥ 0.2 % | |
| **Respiratory sensitizer sub-category 1A** | ≥ 0.1 % | ≥ 0.1 % | |
| **Respiratory sensitizer sub-category 1B** | ≥ 1.0 % | ≥ 0.2 % | |
| **Skin sensitizer Category 1** | -- | -- | ≥ 0.1 % (see note) |
| | -- | -- | ≥ 1.0 % |
| **Skin sensitizer sub-category 1A** | -- | -- | ≥ 0.1 % |
| **Skin sensitizer sub-category 1B** | -- | -- | ≥ 1.0 % |

***NOTE:*** *Some competent authorities may require SDS and/or supplemental labelling only, as described in 3.4.4.2 for mixtures containing a sensitizing ingredient at concentrations between 0.1 and 1.0 % (or between 0.1 and 0.2 % for a gaseous respiratory sensitizer). While the current cut-off values reflect existing systems, all recognize that special cases may require information to be conveyed below that level.*

### 3.4.4 Hazard communication

3.4.4.1 General and specific considerations concerning labelling requirements are provided in *Hazard communication: Labelling* (Chapter 1.4). Annex 1 contains summary tables about classification and labelling. Annex 3 contains examples of precautionary statements and pictograms which can be used where allowed by the competent authority. Table 3.4.5 below presents specific label elements for substances and mixtures that are classified as respiratory and skin sensitizers based on the criteria in this chapter.

**Table 3.4.5: Label elements for respiratory or skin sensitization**

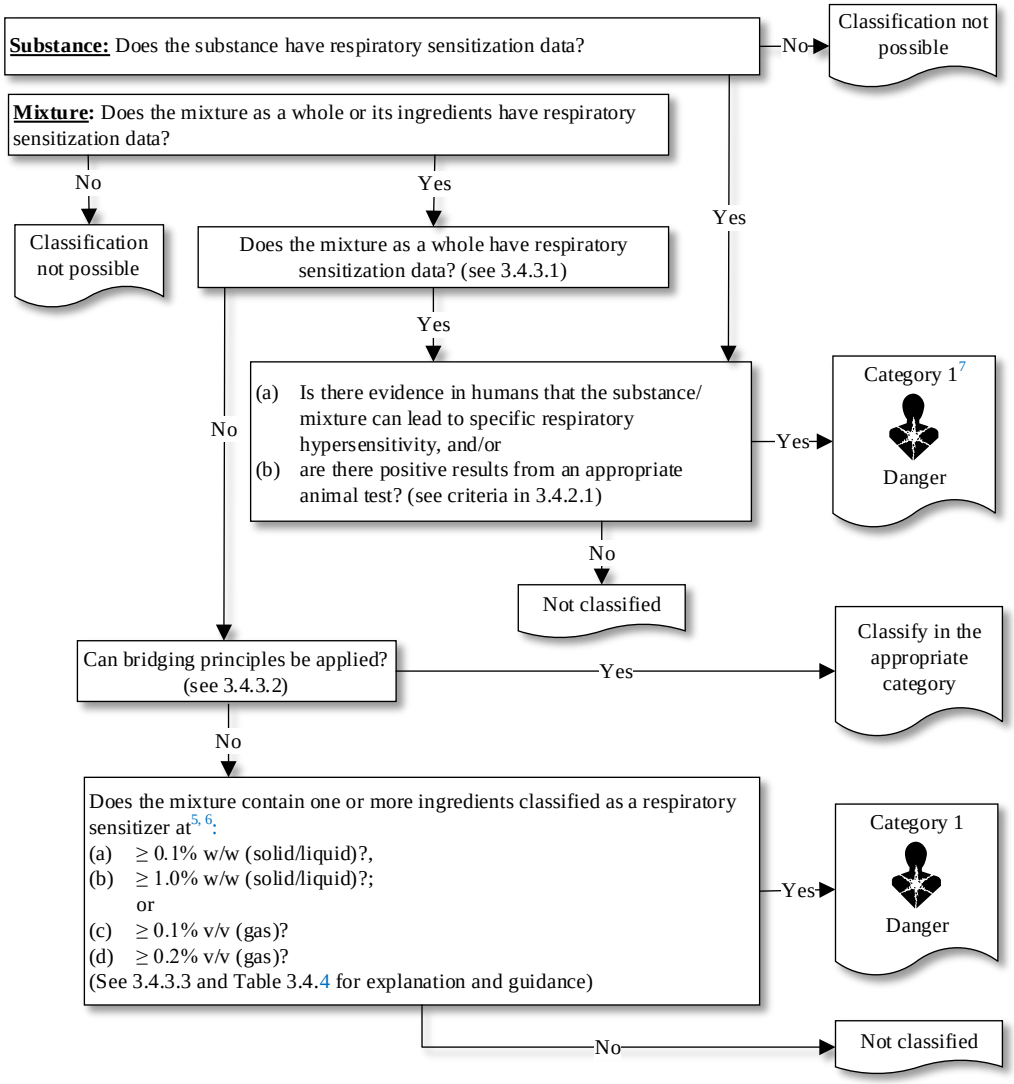
| | **Respiratory sensitization Category 1 and sub-categories 1A and 1B** | **Skin sensitization Category 1 and sub-categories 1A and 1B** |
|---|---|---|
| **Symbol** | Health hazard | Exclamation mark |
| **Signal word** | Danger | Warning |
| **Hazard statement** | May cause allergy or asthma symptoms or breathing difficulties if inhaled | May cause an allergic skin reaction |

3.4.4.2 Some chemicals that are classified as sensitizers may elicit a response, when present in a mixture in quantities below the cut-offs established in Table 3.4.4, in individuals who are already sensitized to the chemicals. To protect these individuals, certain authorities may choose to require the name of the ingredients as a supplemental label element whether or not the mixture as a whole is classified as sensitizer.

### 3.4.5 Decision logic and guidance

The decision logics which follow are not part of the harmonized classification system but are provided here as additional guidance. It is strongly recommended that the person responsible for classification study the criteria before and during use of the decision logics.

### 3.4.5.1 *Decision logic 3.4.1 for respiratory sensitization*

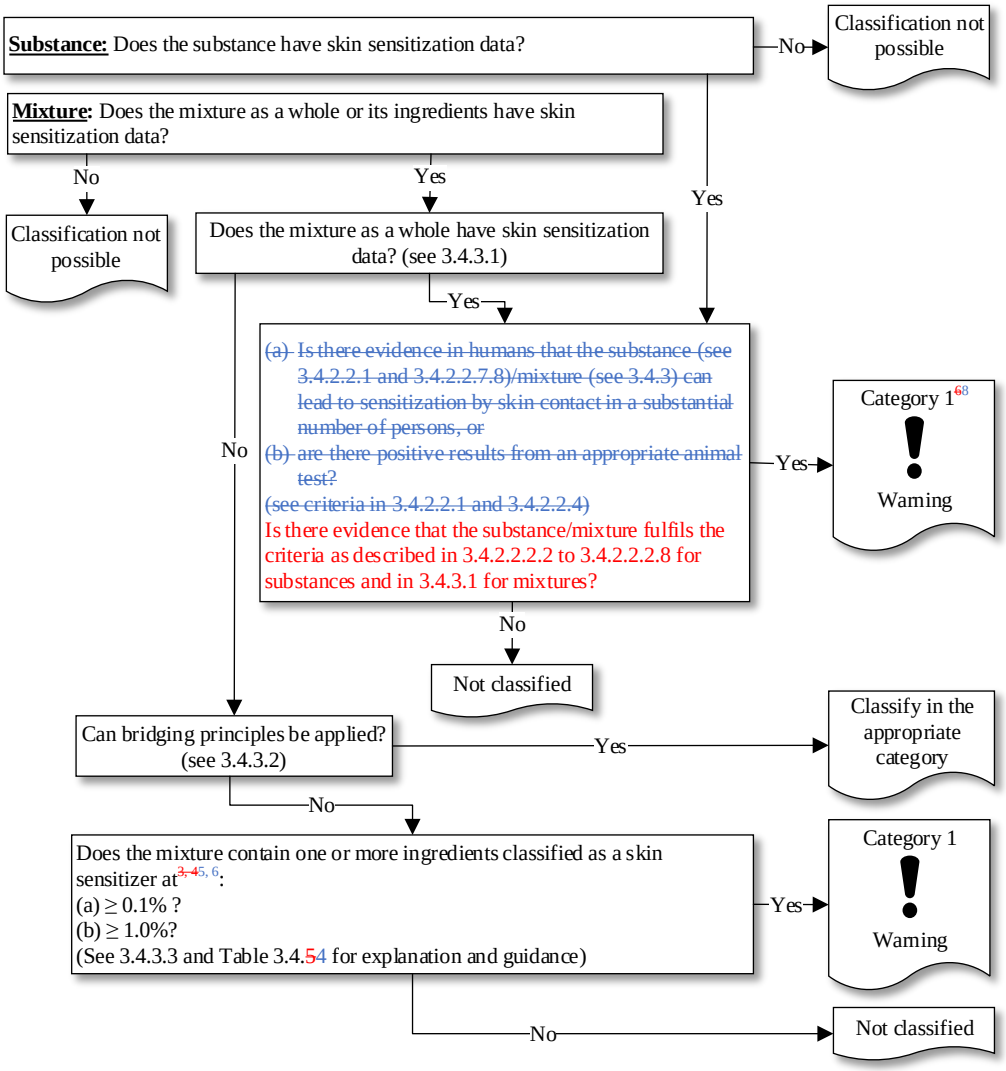



[5]~~3~~ *For specific concentration limits, see "The use of cut-off values/concentration limits" in Chapter 1.3, paragraph 1.3.3.2.*

[6]~~4~~ *See 3.4.4.2.*

[7] ~~5~~ *See 3.4.2.1.1 for details on use of Category 1 sub-categories.*

### 3.4.5.2 *Decision logic 3.4.2 for skin sensitization*




## 3.4.5.3 Background guidance

### 3.4.5.3.1 *Relevant guidance documents*

Mechanistic information on the process of skin sensitization is available in the OECD document on the Adverse Outcome Pathway for skin sensitization (see OECD, (2014)). This information can be helpful in understanding the value of the individual *in chemico* and *in vitro* methods compared to the *in vivo* methods.

### 3.4.5.3.2 *Guidance on the use of human data*

3.4.5.3.2.1 The classification of a substance can be based on human evidence generated from a variety of sources. These sources include human predictive patch testing, epidemiological studies, case studies, case reports or histories, diagnostic patch testing and medical surveillance reports, and poison control centre information. This data may have been generated for consumers, workers, or the general population. When considering human evidence, consideration should be given to the size, exposure level, and exposure frequency of the exposed

[53] *For specific concentration limits, see "The use of cut-off values/concentration limits" in Chapter 1.3, paragraph 1.3.3.2.*
[64] *See 3.4.4.2.*
[85] *See 3.4.2.2.1 for details on use of Category 1 sub-categories.*

population. Guidance for evaluating human evidence and the criteria in 3.4.2.2.2 ~~are~~ is provided by some competent authorities (e.g., ECHA Guidance on the Application of the CLP Criteria, 2017).

3.4.5.3.2.2 Positive data from predictive patch testing (HRIPT or HMT) conducted through human experimental and clinical studies, showing allergic contact dermatitis caused by the test substance can be used to classify substances for skin sensitization These studies are generally conducted in controlled clinical settings and in general the larger the population size, the more reliable the study outcome is. Criteria for evaluating this data are provided in paragraph 3.4.2.2.2.1 and 3.4.2.2.2.

3.4.5.3.2.3 Positive data from well-run epidemiological studies (in accordance with WHO ~~COIMS~~ CIOMS guidelines, 2009) can be used for classifying substances for skin sensitization. Some examples of epidemiological studies may include case control studies, cohort studies, cross-sectional studies, or longitudinal studies. These studies should have large sample sizes with well-documented exposures to a substance.

3.4.5.3.2.4 A specific type of epidemiological study (such as randomized control studies or trials) may include information from diagnostic patch testing. Diagnostic patch testing is considered by some competent authorities to be the gold standard in diagnosing contact allergy in dermatitis patients (Johansen et al, 2015). Importantly, due consideration needs to be given to the appropriate selection of vehicle, substance and patch test concentrations for the purpose of not causing false negatives, false positives, irritant reactions or inducing contact allergy (skin sensitization). Positive data from experimental/clinical/diagnostic studies in humans and/or well-documented episodes of allergic contact dermatitis may be used to classify substances for skin sensitization, when it can be assumed with sufficient likelihood that the tested substance was indeed the most likely cause for induction of sensitisation. Therefore, it should be established that there is at least a general likelihood that the respective patient(s) had been previously exposed to the substance. On the other hand, negative results from such tests are not sufficient to prove that the test substance should not be classified as a skin sensitizer.

3.4.5.3.2.5 Human data not generated in controlled experiments with volunteers for the purpose of hazard classification (e.g. case studies, case reports and case histories, and poison control centre information) can be used with caution. Consideration should be given to the frequency of cases, the inherent properties of the substances, as well as factors such as the exposure situation, bioavailability, individual predisposition, cross-reactivity and preventive measures taken.

3.4.5.3.2.6 Special consideration should be given to negative human data as full dose-response information is generally not available. For example, a negative result in an HRIPT or HMT at a low concentration may not allow for the conclusion that the substance does not have skin sensitizing properties as such effect at a higher concentration may not be excluded. In addition, negative human data should not necessarily be used to negate positive results from animal studies and/or defined approaches, but can be used as part of a weight of evidence assessment. For both animal and human data, consideration should be given to the impact of the vehicle (e.g. Wright et al, 2001 and Kligman, 1966).

3.4.5.3.2.7 For example, negative results from substances tested in a predictive patch test at a DSA (dose per skin area) of < 500 μg/cm$^2$ imply that a classification for skin sensitization might not be needed at all, however, classification as category 1A or 1B cannot be ruled out, because the concentration tested was not high enough to exclude these possibilities. The same holds for test results for which it is unknown whether the test concentration corresponded to a DSA < 500 μg/cm$^2$. Negative results from substances tested at a DSA ≥ 500 μg/cm$^2$ suggest that classification might not be needed. ~~but~~However, while classification as category 1A can be ruled out, classification as category 1B cannot, because a higher test concentration might have resulted in a positive test result. However, a negative test result at a concentration of 100% ~~would indicate that~~ can justify no classification (based on this test). ~~However~~Nevertheless, negative results at low concentrations may be informative for mixtures containing the substance at similar and lower concentrations.

3.4.5.3.3 *Guidance on the use of standard animal data*

~~A positive result in a guinea pig test is defined as a score above zero according to the applicable grading scale such as the Magnusson and Kligman grading scale for OECD Test Guideline 406 at one or more of the two observations. A score of 0.5, which is sometimes reported, is therefore also considered a positive result.~~

3.4.5.3.3.1 The most common assays used for dermal sensitization testing in animals are the Local Lymph Node Assay (LLNA, OECD Test Guidelines 429 and 442A and 442B), the Guinea Pig Maximization Test (GMT, OECD Test Guideline 406) and the Buehler test (OECD Test Guideline 406). When evaluating the quality of the study, consideration should be given, as relevant, to the strain of the mouse and guinea pig used, the number, age, and sex of the animals, and the test conditions used (e.g., preparation of patch test site, dose level selection, chemical preparation, positive and negative test controls).

3.4.5.3.3.2 OECD test guidelines for the LLNA include the radioactive assay (OECD Test Guideline 429) and non-radioactive assays (OECD Test Guideline 442A and 442B; LLNA:DA, LLNA:BrdU-ELISA, and LLNA:BrdU-FCM). In these tests, sensitisers are characterised by increasing the group mean Stimulation Index (SI, a measure of lymph node proliferation) in treated groups vs. concurrent vehicle controls by more than a predefined critical value which is different for each form of the LLNA (e.g., SI ≥ 3 for the radioactive LLNA, SI ≥ 1.6 for the LLNA:BrdU-ELISA). For sensitisers, sub-categorization is performed based on the effective concentration (EC) causing an increase in SI of exactly the critical magnitude (e.g. the EC3 under OECD Test Guideline 429 is the concentration leading to an exactly threefold increase in group mean SI vs. control).

3.4.5.3.3.3 The respective OECD Test Guidelines for the different LLNA variants specify that a pre-screen test should be undertaken to determine the highest concentration to be tested. If such a test has not been performed and the LLNA was carried out with a test concentration < 100%, a rationale (e.g. based on solubility, local or systemic toxicity, see OECD Test Guidelines 429, and 442A and 442B) needs to be provided that the highest test concentration represents the maximum testable concentration. Otherwise, the reliability of a negative test result has to be considered compromised.

3.4.5.3.3.4 EC values are normally obtained by interpolation between adjacent test concentrations, i.e. between the highest test concentration causing an SI below, and the lowest test concentration causing an SI above the critical value. However, care must be taken when the EC value falls below the lowest concentration tested and can therefore only be estimated by extrapolation, which is associated with additional uncertainty. In some cases, the SI at the highest concentration tested falls only slightly below the critical SI value, which raises the question of upward extrapolation (unless the maximum testable concentration has been applied). These and other issues regarding the reliability of LLNA results are further discussed in Ryan et al. (2007) and Annex 3 of OECD Series on Testing and Assessment No. 336 (Supporting Document to OECD Guideline Document 497), which also provides a highly curated database of Test Guidelines 429 LLNA EC3 values.

3.4.5.3.3.5 Further limitations have been identified for the radioactive and non-radioactive LLNAs. For example, substances containing certain functional groups may interfere with the accuracy of the assay. These limitations as well as the possibility of borderline positive results are described in OECD Test Guidelines 429, and 442A and 442B. Variability in EC values for the same substance may also be the result of the vehicle used. For example, analysis has shown an underestimation of potency (i.e., higher EC3 values) with predominantly aqueous vehicles or propylene glycol (see Jowsey, 2008).

3.4.5.3.3.6 For OECD Test Guideline 406, the concentration of test chemical used for each induction exposure should be systemically well-tolerated using the highest dose to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest non-irritant dose. A positive result in a guinea pig test is defined as a grade above zero according to the applicable grading scale such as the Magnusson and Kligman grading scale for OECD Test Guideline 406 at one or more of the two observation time-points. A grade of 0.5, which is sometimes reported, is therefore also considered a positive result.

3.4.5.3.4 *Guidance on the use of defined approaches*

Defined approaches validated according to international procedures and described in OECD Guideline 497 have been characterized for the level of confidence that can be assigned to the predictions based on the applicability domain of the individual information sources used and the Data Interpretation Procedure applied (see Table 3.4.6). Other defined approaches under consideration but not yet validated according to international procedures and described in OECD Guidance Document 256 according to internationally agreed criteria for their reporting (OECD Guidance Document 255) may be accepted by some competent authorities.

3.4.5.3.5 *Guidance on the use of non-stand-alone in chemico/in vitro methods*

Individual *in chemico/in vitro* methods such as those reported in OECD Test Guidelines 442C, 442D and 442E, due to their limited mechanistic coverage, cannot be used on their own to conclude on Category 1 or no classification according to the criteria defined in Table 3.4.7 and further data are necessary for classification in Tier 2. In addition, although some of these methods provide~~s~~ quantitative information, these cannot be used for the purposes of subcategorization into sub-categor~~y~~ies 1A and ~~subcategory~~ 1B since the criteria have not been validated according to international procedure. Nevertheless, such quantitative information may be accepted by a competent authority when used in a weight of evidence assessment under Tier 2 for the purpose of subcategorization. This is also in line with the statement in these Test Guidelines that "Depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into Category 1." Therefore, the GHS also allows a competent authority to decide that a positive result with one of these non stand-alone *in chemico/in vitro* methods, may be used on its own to classify in category 1 and whether Test Guideline 442C (A~~a~~ppendix III) kinetic Direct Peptide Reactivity Assay (kDPRA) can be used to differentiate between category 1A and no category 1A.

3.4.5.3.6 *Guidance on the use of non-standard data*

3.4.5.3.6.1 Validated but not yet adopted *in chemico/in vitro* methods such as those reported under ~~3.4.5.3.6.1~~ 3.4.5.3.6.2 as well as *in vivo* test methods which do not comply with internationally agreed guidelines for the identification of skin sensitizers or the assessment of skin sensitizing potency may provide supportive evidence when used in an overall weight of evidence assessment (i.e. Tier 3).

3.4.5.3.6.2 A non-exhaustive list of other validated *in chemico/in vitro* test methods accepted by some competent authorities but not adopted as OECD Test Guidelines is provided below. A competent authority may decide which classification criteria, if any, should be applied for these test methods:

(a) The Genomic Allergen Rapid Detection (GARD)potency is a transcriptomics-based *in vitro* assay addressing the third key event of the skin sensitization Adverse Outcome Pathway (activation of dendritic cells) similar to the GARDskin but uses a different gene signature that provides sub-categorization of skin sensitizers (Gradin et al., 2020; Zeller et al., 2017; Corsini et al. 2021).

(b) The SENS-IS assay is a genomic approach applied to a Reconstructed Human Epidermis (RHE) (Cottrez et al., 2015; Cottrez et al., 2016).

(c) The Epidermal Sensitization Assay (EpisensA) is based on the measurement of the upregulation of four genes in a reconstructed human epidermis (RhE) to discriminate between sensitisers and non-sensitisers (Saito et al., 2017).

3.4.5.3.7 *Guidance on the Weight of Evidence assessment for classifying substances and mixtures for skin sensitization*

~~In some situations where several results from test or non-test methods are available and in disagreement with each other with respect to the classification outcome, the tiered approach to classification for skin sensitisation requires a weight of evidence assessment.~~

3.4.5.3.7.1 There may be situations where results from tests and/or non-test methods are available but disagree with each other with respect to the classification. In these situations, the tiered approach to classification for skin sensitization requires a weight of evidence assessment consistent with the principles elaborated in sections 1.3.2.4.2 and 1.3.2.4.9 on test data quality and weight of evidence, respectively. In addition, some guidance on the weight of evidence assessment specific for skin sensitisation is provided below which can be applied when the general principles do not result in a conclusion on the classification. It should be noted that human and animal results for a substance obtained at low concentrations may still be informative for classifying a mixture containing the substance at similar or lower concentrations.

3.4.5.3.7.2 Mutual compatibility of study results

3.4.5.3.7.2.1 In cases where results are in disagreement with each other (e.g., not classified vs. category 1, sub-category 1A or 1B; sub-category 1A vs. 1B), a weight of evidence assessment becomes necessary. However, less obvious situations may also occur such as where certain studies may point to not classified or sub-category 1B, while it cannot be excluded that a stricter classification might have resulted under a different dosing regime. For example, a negative HMT result at a dose per skin area of 100 µg/cm$^2$ cannot exclude that a positive result

might have been obtained at e.g., 300 µg/cm$^2$ (sub-category 1A) or 700 µg/cm$^2$ (sub-category 1B). The same holds for LLNA test results obtained from tests which have not been carried out using the highest possible test concentration (see OECD test guideline 429 for details).

3.4.5.3.7.2.2 In the following ambiguous cases, study results for substances and mixtures would not be in disagreement with another study result pointing at that stricter classification:

(a) A not classified result obtained at a lower test concentration does not exclude the possibility of a sub-category 1B outcome at a higher test concentration. Therefore, a not classified result obtained at a low concentration is compatible with other not classified outcomes, or with category 1 and sub-category 1B outcomes obtained at higher test concentrations.

(b) A not classified result at a very low-test concentration does not even exclude a possible outcome of sub-category 1A at a higher test concentration. Therefore, a not classified outcome obtained at a very low-test concentration is compatible with all possible classification outcomes (i.e., not classified, category 1, sub-category 1A or 1B) obtained at higher test concentrations.

(c) A sub-category 1B result at a higher test concentration does not exclude a sub-category 1A outcome at a lower test concentration. Therefore, a Category 1B classification tested at a high-test concentration is compatible with other outcomes of sub-category 1B, or even sub-category 1A, obtained at lower test concentrations.

3.4.5.3.7.2.3 If at least one unambiguous study result allows for sub-categorisation of a substance or mixture and all other study results are not in disagreement (see above), then it can be classified into a sub-category. For example, if all study results are in the same sub-category (i.e., sub-category 1A or 1B), or with at least one study permitting sub-categorisation (i.e., either sub-category 1A or 1B) and all other studies classified into category 1 without sub-categorisation, then the substance or mixture can be sub-categorised.

3.4.5.3.7.3 Weight of evidence considerations for giving one study result more weight than another

3.4.5.3.7.3.1 Some classifiers or competent authorities may take various approaches to evaluate study results given the required level of expert judgement (see 1.3.2.4.8) required to perform a weight of evidence assessment. Competent authorities may specify their preferred approach in their own guidance. For example, through:

(a) Applying a precautionary approach, giving more weight to studies resulting in the stricter classification outcome.

(b) Giving human data higher weight than animal or non-test data.

(c) Giving certain animal data (e.g., LLNA data) more weight than other animal data (e.g., Buehler test data).

3.4.5.3.7.3.2 Often, several results (of the same or different type) may have to be considered in the weight of evidence assessment. There are no generally recognised rules for this situation, however, possible solutions to integrating several results of the same type may include, for example:

(a) A precautionary approach where the strictest classification outcome from all studies of sufficient quality is assigned as the overall classification outcome.

(b) Averaging the obtained dose descriptors (e.g., LLNA EC3 values) or classification outcomes (no classification, Category, 1, 1A, 1B). A detailed discussion of such approaches can be found in Annex 3 (on LLNA data) and Annex 4 (on HMT/HRIPT data) of OECD Series on Testing and Assessment No. 336 (Supporting Document to OECD Guideline Document 497).

## Table 3.4.6: Criteria for defined approaches

| Category | OECD Guideline 497 on Defined Approaches for Skin sensitization<br>"2 out of 3" (2o3) defined approach | OECD Guideline 497 on Defined Approaches for Skin sensitization<br>Integrated testing strategy (ITSv1) defined approach and<br>Integrated testing strategy (ITSv2 defined approach) |
|---|---|---|
| | **2o3** defined approach to skin sensitization hazard identification based on *in chemico* (key event 1 - Direct Peptide Reactivity Assay (KE1-DPRA)) and *in vitro* (key event 2-OECD 442D Appendix IA, key event 3 - human Cell Line Activation Test (KE3-h-CLAT))<br><br>Assays are run for two key events, and if these assays provide consistent results, then the chemical is predicted accordingly as sensitizer or non-sensitizer. If the first two assays provide discordant results, the assay for the remaining key event is run. The overall result is based on the two concordant findings taking into account the confidence on the obtained predictions as described in the Guideline | **ITSv1** based on *in chemico* (KE1-DPRA) and *in vitro* (KE3-h-CLAT) data, and *in silico* (Derek Nexus) predictions.<br>**ITSv2** based on *in chemico* (KE1 -DPRA) and *in vitro* (KE3 -h-CLAT) data, and in silico (OECD QSAR Toolbox) predictions.<br><br>Quantitative results of h-CLAT and DPRA are converted into a score from 0 to 3. For the *in silico* prediction (Derek or OECD QSAR ToolBox), a positive outcome is assigned a score of 1; a negative outcome is assigned a score of 0. When these scores have been assessed, a total battery score ranging from 0 to 7, calculated by summing the individual scores, is used to predict the sensitizing potential (hazard identification; GHS Cat. 1 vs. no classification) and potency (GHS Cat. 1A, Cat. 1B and no classification). |
| **1** | 2 out of 3 or 3 out of 3 positive predictions | Total battery score ≥ 2 |
| **1A** | Not applicable | Total battery score 6-7 |
| **1B** | Not applicable | Total battery score 2-5 |
| **Not classified** | 2 out of 3 or 3 out of 3 negative predictions | Total battery score < 2 |

## Table 3.4.7: Criteria for individual *in chemico/in vitro* methods

| Category | OECD Test Guideline 442C<br>Key event-based Test Guideline for *in chemico* skin sensitization assays addressing the adverse outcome pathway (AOP) Key Event on covalent binding to proteins | | | OECD Test Guideline 442D<br>Key event-based Test Guideline for *in vitro* skin sensitization assays addressing the AOP Key Event on keratinocyte activation antioxidant response element-nuclear factor-erythroid 2-related factor 2 (ARE-Nrf2) luciferase methods | | OECD Test Guideline 442E<br>In vitro skin sensitization assays addressing the AOP Key Event on activation of dendritic cells | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Method described in Appendix I**<br><br>**The Direct Peptide Reactivity Assay (DPRA)[a]** | **Method described in Appendix II**<br><br>**The Amino acid Derivative Reactivity Assay (ADRA) [a]** | **Method described in Appendix III**<br><br>**The kinetic Direct Peptide Reactivity Assay (kDPRA)[b]** | **Method described in Appendix 1A[a]** | **Method described in Appendix 1B**<br><br>**Lusens [a]** | **Method described in Annex I**<br><br>**human Cell Line Activation Assay (h-CLAT) [a]** | **Method described in Annex II**<br><br>**U937 Cell Line Activation Test [a]** | **Method described in Annex III**<br><br>**Interleukin-8 luciferase (IL-8 Luc) assay [a]** | **Method described in Annex IV**<br><br>**Genomic Allergen Rapid Detection for assessment of skin sensitizers [a]** |
| | Methods: in chemico methods addressing the process of haptenation by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (DPRA and kDPRA) or towards model synthetic amino acid derivatives containing either N-(2-(1-naphthyl) acetyl)-L-cysteine (NAC) or α-N-(2-(1-naphthyl) acetyl)-L-lysine (NAL) (ADRA).<br>The criteria are based on the mean of cysteine and lysine peptides percent depletion (DPRA), kinetic rates of cysteine depletion (kDPRA) and mean NAC and NAL percent depletion value (ADRA). Predictions models based on the cysteine or NAC percent depletion value alone in case the unreacted lysine peptide or NAL cannot be reliably measured can be applied for the DPRA and ADRA. | | | Methods: cell-based methods addressing the process of keratinocytes activation, by assessing with the help of luciferase, the Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes following exposure of the cells to the test chemical.<br>Cell viability is quantitatively measured in parallel by enzymatic conversion of the dye 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).<br>The criteria are based on the induction of the luciferase gene above a given threshold, quantified at subtoxic concentrations. Criteria should be met in 2 of 2 or in 2 of 3 repetitions. | | Methods: cell-based methods addressing the process of monocytes/dendritic cell activation by either quantifying the change in the expression of cell surface marker(s) (e.g. cluster of differentiation 54 (CD54), cluster of differentiation 86 (CD86)) or the change in IL-8 expression or the transcriptional patterns of an endpoint-specific genomic biomarker signature following exposure of the cells to the test chemical.<br><br>Criteria should be met in 2 of 2 or in at least 2 of 3 repetitions for test methods described in Annexes I, II and III or in three valid biological replicates for test method described in Annex IV. | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **1** | The mean cysteine/lysine % depletion > 6.38%<br>Or<br>the mean cysteine % depletion >13.89% | The mean NAC and NAL % depletion ≥ 4.9%<br>Or<br>NAC% depletion ≥ 5.6% | Not applicable | The following 4 conditions are all met in 2 of 2 or in the same 2 of 3 repetitions:<br>1. Imax equal or higher than (≥) 1.5 fold and statistically significantly different to the solvent control<br>2. The cellular viability is higher than (>) 70% at the lowest concentration with induction of luciferase activity equal or above 1.5 fold<br>3. The $EC_{1.5}$ value is less than (<) 1000 µM (or < 200 µg/mL for test chemicals with no defined molecular weight)<br>4. There is an apparent overall dose-dependent increase in luciferase induction | The following conditions are all met in 2 of 2 or in the same 2 of 3 repetitions:<br>1. A luciferase induction above or equal to (≥) 1.5 fold as compared to the solvent control is observed in at least 2 consecutive non-cytotoxic tested concentrations (i.e. cellular viability is equal or higher than (≥) 70%)<br>2. At least three tested concentrations should be non-cytotoxic (cellular viability equal or higher than (≥) 70%). | At least one of the following conditions is met in 2 of 2 or in at least 2 of 3 independent runs:<br>The Relative Fluorescence Intensity of CD86 is equal to or greater than 150% at any tested concentration (with cell viability ≥ 50%)<br>or<br>the Relative Fluorescence Intensity of CD54 is equal to or greater than 200% at any tested concentration (with cell viability ≥ 50%). | The following condition is met in 2 of 2 or in at least 2 of 3 independent runs:<br>The stimulation index of CD86 is equal or higher (≥) than 150% and/or interference is observed | The induction of normalised interleukin-8 luciferase activity (Ind-IL8LA) is equal or higher than (≥) 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA is equal or higher than (≥) 1.0 in at least 2 out of a maximum of 4 independent runs | The mean Decision Value (DV) is ≥0 |
| 1A | Not applicable | | log kmax ≥ -2.0 | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |
| 1B | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |
| Not classified | The mean cysteine/lysine % depletion ≤ 6.38%<br>or<br>the mean cysteine % depletion ≤ 13.89 % | The mean NAC and NAL % depletion < 4.9%<br>Or<br>NAC% depletion < 5.6% | Not applicable | At least one of the conditions for Category 1 is not met | At least one of the conditions for Category 1 is not met | None of the conditions for Category 1 is met | The stimulation index of CD86 is < 150% at all non-cytotoxic concentrations (cell viability ≥ 70%) and if no interference is observed | The Ind-IL8LA is less than (<) 1.4 and/or the lower limit of the 95% confidence interval of Ind-IL8LA is less than (<) 1.0 in at least 3 out of a | The mean Decision Value (DV) is <0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | maximum of 4 independent runs | |

[a] *Data cannot be used as stand-alone to conclude on classification in Category 1 or on no classification in tier 1 but could be used to conclude on classification in category 1 in Tier 2 depending on the decision of the competent authority for their regulatory framework.*

[b] *A competent authority may decide that data can be used as stand-alone to conclude on classification in sub-category 1A.*

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