REPORT FROM THE CHEMICALS SELECTION SUB-COMMITTEE TO THE MANAGEMENT TEAM ON POTENTIAL REASONS FOR THE MISCLASSIFICATION OF CHEMICALS IN THE EPISKIN AND EPIDERM ASSAYS

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1. INTRODUCTION

The ECVAM Skin Irritation Validation Study (SIVS) was designed to evaluate the abilities of several *in vitro* tests, including the two commercially available tests EPISKIN and EpiDerm, to predict the presence and absence of skin irritation potential, according to the EU classification scheme. On completion of the experimental phase of the validation study, analysis of the *in vitro* and *in vivo* data indicated that 22 (1 chemical is kept confidential in this analysis upon specific request by the company) out of 60 chemicals were incorrectly classified by EPISKIN and/or EpiDerm in at least one of the participating laboratories (having 3 valid runs), when defined *in vitro* prediction models were used to extrapolate the *in vitro* data to the animal-based classifications. Non-valid runs were not taken into account because misclassification could be due to technical issues.

The Management Team of the study expressed the need to rationalize the falsely classified chemicals.

Thus, one of the resulting actions of the 4th Management Team meeting held at Ispra, Italy, on 12-13 July 2005 was, that the Chemicals Selection Sub-Committee (CSSC) should prepare a report on potential reasons for misclassification and/or non-qualification of chemicals tested in the SIVS.

With this scope, the CSSC prepared this first analysis in which the following points were investigated (with the name of the person responsible for the respective analysis indicated in parentheses):

1. range of the *in vivo* data (Sebastian Hoffmann),
2. physical-chemical and structural properties (Tatiana Netzeva, Grace Patlewicz, Ana Gallegos Saliner & Andrew Worth),
3. physical-chemical properties (Tom Cole),
4. potential influences of other human health effects such as skin sensitisation (R43) or eye irritation (R41 and R36; Chantra Eskes) and;
5. relevant observations made by the participating laboratories (Chantra Eskes).

On 14 December 2005, the CSSC invited comments from the six laboratories participating in Phase II, relevant to observed experimental anomalies.

Concise lists of observations were received from L'Oréal and Sanofi, whereas the other laboratories provided many comments relating to various observations, as e.g., occasional apparent interaction of a chemical with the rubber or teflon inlay of the vial lid; need for occasional removal of the mesh adhered to the tissue using tweezers or; occurrences of white specks on tissues from certain batches after MTT assay; only to mention a few.

Because of the heterogeneous nature of the comments received by the laboratories and in order to focus this preliminary investigation on its primary aim (i.e., to analyse the potential influence of the *in vivo* scores, physical–chemical properties, structure-activity relationships, other risk phrases and stability on the misclassification of chemicals) the CSSC decided not to include any observations related to biological effects and/or possible interactions with the tissue model and the MTT assay. Investigation of biochemical observations would require technical expertise in the evaluated assays.

2. RANGE OF THE *IN VIVO* DATA

Crucial criteria for the selection of chemicals in SIVS were the availability and quality of the *in vivo* data. It was agreed that a substance had to be registered in either the New Chemicals Database (NCD), the ECETOC-database for skin irritation or in the TSCA-database. Among other criteria, in general, Draize scores had to be available from each of at least three rabbits. However, substances were also considered when mean scores were reported for tests with more than three rabbits (e.g., certain NCD chemicals). Furthermore, the *in vivo* data had to comply with the current European classification scheme (no label vs. R38). Thus, it was assured that the skin irritation classification for each chemical corresponded to the respective *in vivo* data.

Review of individual Draize scores allows insight on the correlation of the *in vivo* data to the assigned classifications. The degree of confidence in a classification based on the *in vivo* data would vary according to the consistency of the reported scores and their numerical difference from the conventional threshold value of 2, differentiating irritants from non-irritants. For example, a chemical with Draize scores of 0 for both dermal effects (erythema and oedema) in all animals would be classified as non-irritant with greater confidence than a chemical with near threshold (i.e., 2) scores of 1.7 for both effects in all animals. In addition, as sample sizes are restricted, random sampling might result in alternative classifications. For example, a chemical with four available scores for the same effect (e.g., erythema: 1.7, 2.0, 0.7, 2.0; median: 1.7) would qualify as non-irritant (i.e., median score < 2) according to convention. Equally according to convention, if either of the scores 1.7 or 0.7 were not available or not reported, the same chemical would qualify as irritant (i.e., two out of three scores ≥ 2 for the same dermal effect).

For each of the SIVS Phase II chemicals the Draize scores were characterized by a dominant median score, corresponding to the more significant observation effect (erythema or oedema). Illustrated as a graphical distribution (Fig. 2.1) including the respective ranges, dominant median scores are shown separately for non-irritants (upper plot) and irritants (lower plot).

Figure 2.1 (upper plot) indicates five chemicals (10, 17, 21, 33, 50) with dominant median Draize scores of about 1.7, but with maxima up to 2. Table 2.1 (upper section) lists the names of the five chemicals, including dominant dermal effect. Chemicals 17 and 21, with erythema as dominant effect, also produced oedema reaction up to 1.7. Chemicals 10 and 33, also with erythema as dominant effect, showed oedema scores with a maximum of 0.7. Chemical 50 induced borderline erythema reactions in two animals, but the third rabbit did not show any reaction. While these five chemicals qualify as non-irritants (no label) according to the EU classification scheme, occurrence of the borderline erythema scores, coupled to perceptible oedema effects, indicates potential for justifiable misclassification as false positives by the *in vitro* assays.

Similarly, Figure 2.1 (lower plot) indicates four chemicals (31, 49, 51, 60) with dominant median Draize scores of 2, but with maxima also of 2. Table 2.1 (lower section) lists the names of the four chemicals, including dominant dermal effect. Other chemicals with dominant median Draize scores of 2 are evident but which also show maxima greater than 2. Classifications assigned to the latter chemicals (i.e., with single rabbit reactions larger than 2 for at least one dermal effect) were considered as sound. While the four listed chemicals qualify as irritants (R38) according to the EU classification scheme, the borderline dominant median and maximum scores, coupled to insignificant reaction in the respective subordinate

effect, indicates potential for justifiable misclassification as false negatives by the *in vitro* assays.

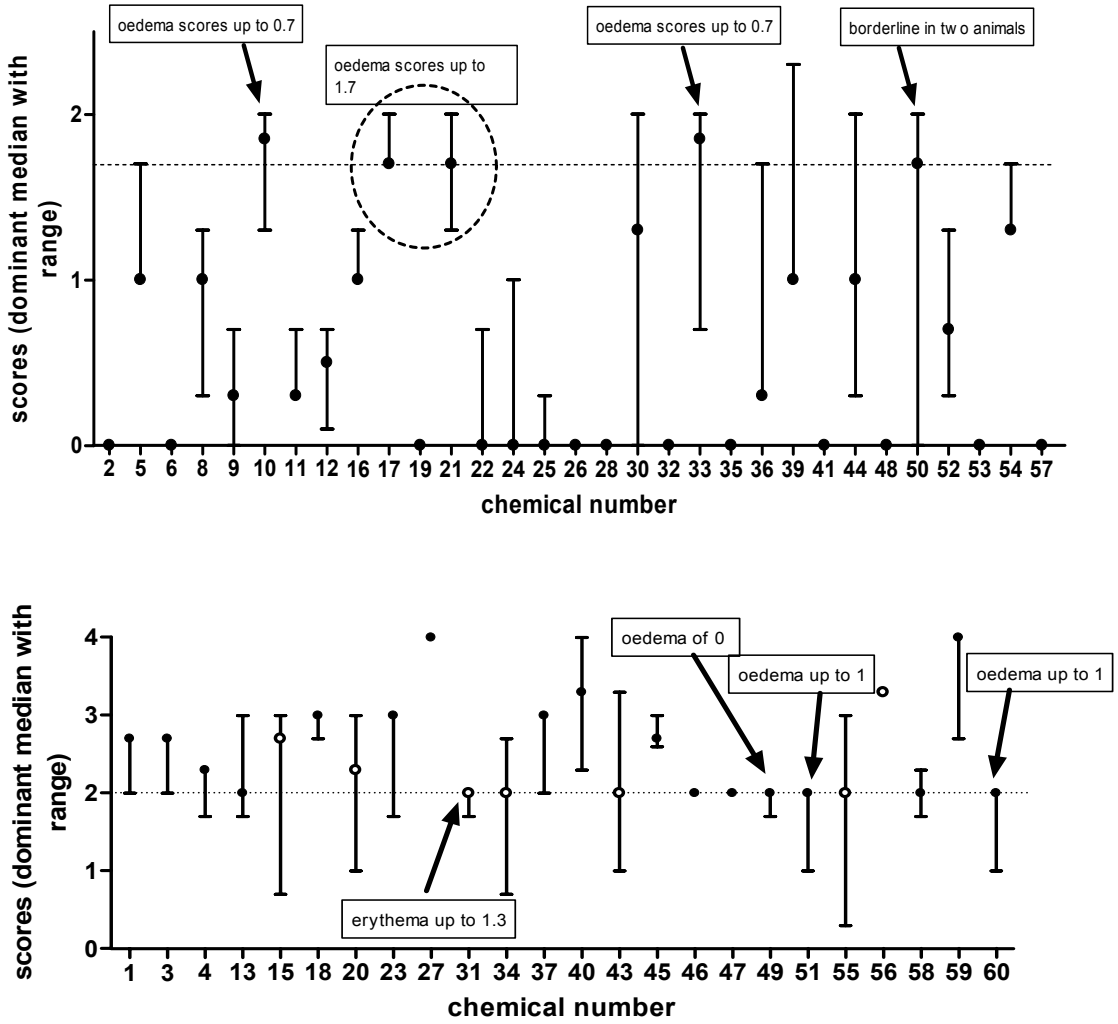


Fig. 2.1. Dominant median Draize scores and range limits for SIVS Phase II chemicals. Erythema dominant effect: dots. Oedema dominant effect: open circles. Chemical numbers refer to the sequential order assigned to the Phase II selection (Appendix 1).

In vitro results

Of the five chemicals with potential for misclassification as false positives *in vitro* (10, 17, 21, 33, 50) only chemical 17 was classified as an irritant in all laboratories, while chemicals 10, 21, 33 and 50 were uniformly and correctly predicted as non-irritant. Consequently, chemicals 10, 21, 33 and 50 were not included among the 22 chemicals for which other potential reasons for misclassification were investigated.

Of the four chemicals with potential for misclassification as false negatives *in vitro* (31, 49, 51, 60) only chemicals 49 and 51 were consistently and incorrectly predicted as negative, while 31 (NQ at IIVS) and 60 were correctly predicted as positive in at least four laboratories (except L'Oréal and Sanofi).

Beside chemical 49, there were three other chemicals (13, 23, 62 [Phase I only]) with irritant erythema scores, but oedema scores of 0, of which chemical 62 was borderline irritant only.

However, possible influence of biochemical factors should be considered before concluding whether this could also be a criterion for potential misclassification relating to *in vivo* data.

Table 2.1. Chemicals with borderline Draize scores, indicating potential for justifiable misclassification by the *in vitro* assays.

SIVS Phase II Chemical N°.	**Decoding N°**	**Chemical Name**	**Database source**	**Dominant dermal effect**
10	28	allyl heptanoate	ECETOC	erythema
17	30	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	NCD	erythema
21	31	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; 5-endo-decylbicyclo[2.2.1]hept-2-ene	NCD	erythema
33	29	heptyl butyrate	ECETOC	erythema
50	27	2-phenylhexanenitrile	NCD	erythema
31	21	A mixture of isomers: ethyl exo-tricyclo[5.2.1.0(2,6)]decane-endo-2-carboxylate; ethyl endo-tricyclo[5.2.1.0(2,6)]decane-exo-2-carboxylate	NCD	oedema
49	20	isostearic acid monoisopropanolamide	NCD	erythema
51	15	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS# 191044-60-7) 1-(3-isopropylphenyl)-1-phenylethane (CAS# 191044-59-4) 1-(4-isopropylphenyl)-1-phenylethane (CAS# 2320-06-1)	NCD	erythema
60	24	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	NCD	erythema

Chemicals 10, 17, 21, 33, 50 (no label, non-irritants): potential for justifiable *in vitro* misclassification as false postives.

Chemicals 31, 49, 51, 60 (R38, irritants): potential for justifiable *in vitro* misclassification as false negatives.

3. QSAR ANALYSIS OF MISCLASSIFIED CHEMICALS

To contribute to the post-hoc rationalization of the data, ECVAM asked the QSAR Group within ECB whether the misclassifications could be explained to some extent on the basis on the underlying physicochemical and/or structural properties of the test chemicals. This report summarises the main findings of the (quantitative) structure-activity relationship ([Q]SAR) analyses.

3.1 Aim

The aim of this work was to apply several (Q)SAR methods to assess whether any explanations for the misclassified chemicals (by EPISKIN and/or EpiDerm) could be offered on the basis of the chemical structure and/or physicochemical properties of the test chemicals. In particular, two approaches were applied:

1) use of physicochemical parameters and calculated descriptors of chemical structure;
2) use of a "weight of evidence" approach based on different QSAR expert systems.

3.2 Use of chemical descriptors

Method

A total of 56 (from 60) chemicals were encoded as simplified molecular input line entry specification (SMILES) strings. Of them, 38 were correctly predicted by both *in vitro* tests (EPISKIN and EpiDerm) and 17 were mispredicted by one or both *in vitro* tests. Three chemicals, a metal complex and 2 mixtures (all mispredicted), were excluded from the analysis, and one chemical failed to generate a SMILES. Initially, several physicochemical descriptors were considered (e.g. mp, bp, vp, ws, log P). In addition, more than 50 chemical descriptors of various types were calculated. Linear discriminant analysis (LDA) was used to derive statistical relationships between the chemical descriptors and the accuracy of the *in vitro* classification. The correctly classified chemicals were indicated with 0, whereas the misclassified chemicals were indicated with 1. In the LDA, the physicochemical properties and calculated descriptors (mainly electrotopological indices) were used as independent variables, one at a time. The electrotopological indices are atom-level descriptors that are calculated for each atom (such as >C<, >N–, =O) or hydride group (such as –CH3, >NH, –OH) in the molecule. Recently, E-state indices for H-atoms have been also developed. Many E-state indices can be interpreted as indicator variables for the presence or absence of defined molecular features. Such interpretations were used in the current project.

Main findings

- The systematic attempt to explain the incorrectly classified *in vitro* chemicals with physicochemical descriptors showed that no single physicochemical descriptor was able to discriminate between correctly and incorrectly predicted *in vitro* skin irritants with accuracy higher than 50%.
- The use of several calculated descriptors allowed identification of some structural trends, although with probably low significance due to the relatively small number of chemicals determining the rules.

Four main clusters (i.e. 4 principal properties) were identified that allow some discrimination between correctly and incorrectly *in vitro* classified chemicals (see Figure 3.1).

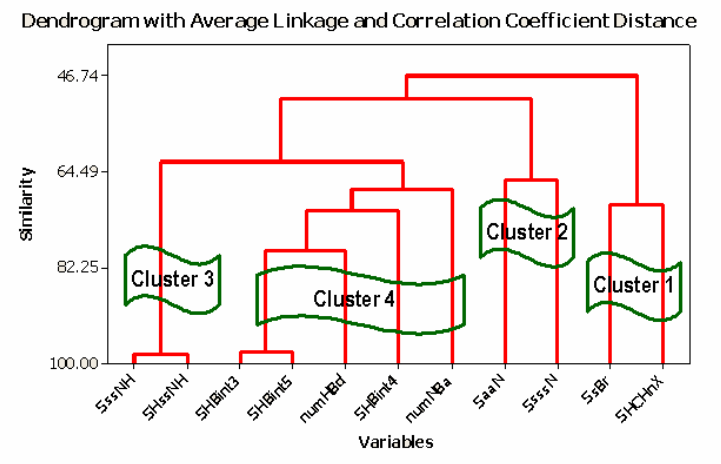
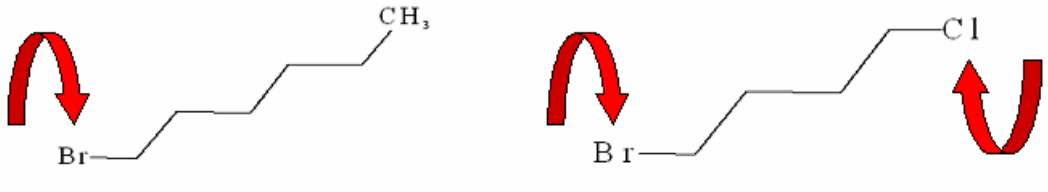
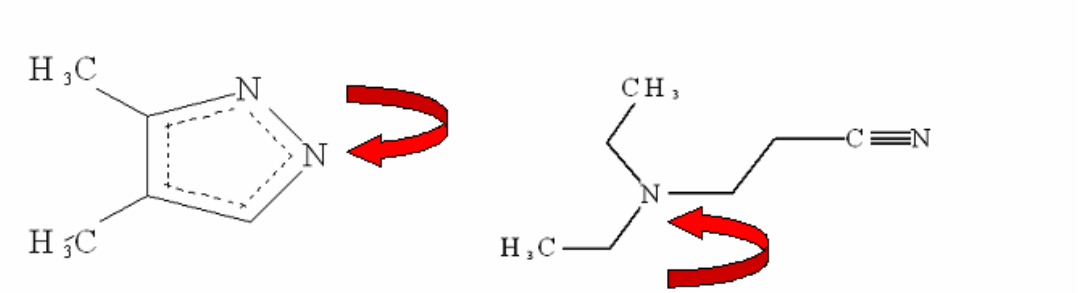


Figure 3.1. A dendrogram showing the grouping of descriptors that demonstrated accuracy higher than 50% in discriminating between correctly and incorrectly classified chemicals on a basis of *in vitro* data.

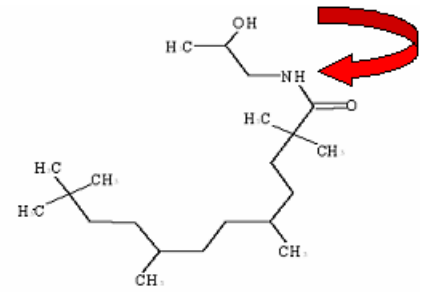
- *Cluster 1* includes SsBr and SHCHnX and accounts for presence of a halogen (Br in particular) in the molecule (e.g. there are 2 chemicals that contain Br – 1-bromohexane and 1-bromo-4-chlorobutane, and both of them are mispredicted).



- *Cluster 2* includes SaaN and SsssN and accounts for presence of N in aromatic heterocycles and of tertiary N-atom (e.g. DMP and 3-diethylaminopropionitrile, both mispredicted).



- *Cluster 3* includes SssNH and SHssNH and accounts for a presence of a secondary aminogroup (e.g. isostearic acid monoisopropanolamide, mispredicted)



- *Cluster 4* is a mixture of chemical features that account for the presence of at least 2 heteroatoms in the molecule, which can participate in a H-bond formation

Conclusions

The overall conclusion is that there is no clear difference in the physicochemical properties between the correctly and incorrectly classified chemicals in the two *in vitro* systems – EPISKIN and EpiDerm, but there might be some structural rationale that for when an incorrect *in vitro* prediction is likely to be made. Thus, it might be possible to identify chemicals that are not correctly predicted by the *in vitro* tests by using a set of structural alerts. However, on the basis of this study, such alerts cannot be identified with statistical significance due to the relatively small number of chemicals investigated. It is also noted that the use of such alerts (on their own) could result in the generation of false positive predictions, i.e. non-irritant chemicals that would be recognised correctly as non-irritant by the *in vitro* test but which would receive a flag for caution by the structural alerts.

3.3 Use of expert systems in weight-of-evidence approach

Method

A total of 60 chemicals were provided with *in vitro* test scores and *in vivo* classifications. SMILES strings were encoded for as many chemicals as possible. One chemical, a metal complex, was excluded from the analysis since a SMILES code could not be derived. Two other compounds were mixtures and the representative chemical structures that could be drawn for these were used in the analysis. In total, there were 21 chemicals (with structures) that were incorrectly predicted by either one or other or both *in vitro* tests. The investigation described below was performed by using the structures for these 21 chemicals.

Several commercial expert systems that are available within ECB were used to derive estimates for these 21 structures. These systems include DEREK, TIMES and the Danish database of (Q)SAR estimates (DK DDB). DEREK is a knowledge-based expert system containing over 320 structural alerts for endpoints such as skin, eye irritation, skin sensitization, mutagenicity, carcinogenicity. TIMES is a hybrid model that encompasses a metabolic simulator for the prediction of skin sensitization hazard and potency. The DK DBB contains (Q)SAR predictions for over 70 models for 166,000 chemicals.

In addition, the Leadscope data mining tool was used to help identify structural analogs with toxicity data that could help in developing read across arguments. This tool is linked to various databases, such as RTECS, FDA CFSAN, and the Carcinogenicity Potency Database (CPDB).

For each misclassified structure, an estimate was made using the three systems. This was then supplemented with any skin/eye irritation data for similar analogs. The resulting matrix of *in silico* predictions was used to provide insights to rationalize the misclassifications.

Main findings

- In many cases the structures that were false negatives in the *in vitro* test did possess alerts for other endpoints (such as irritation, skin sensitization or mutagenicity) and analogs were identified with positive irritation data in either skin, eye or both. In other words, it appears that some of the false negatives generated by the *in vitro* tests could be correctly classified by applying a weight-of-evidence approach based on multiple experts systems and read-across considerations.
- In some cases there was insufficient information to make a judgment.
- In one case there appeared to be a conflict in the trend expected – an alkyl halide classified as positive *in vivo* possessed various structural alerts and analogs with positive irritant data but the disubstituted alkyl halide which possessed the same alerts was classified as a non irritant *in vivo*.

Conclusions

Overall QSARs from the expert systems and use of read across analogs can provide some interesting insights that could be used to build up a weight of evidence before commencing testing. This is relevant to the development of tiered testing strategies for skin irritation.

The expert systems investigated in this study were found to be good at predicting likely irritants. They were less capable of predicting non-irritants. This can be attributed to the nature of some expert systems, which are built on knowledge and data biased for positive (adverse) effects. Rule based systems are prone to predict a high number of false positives since some rules can be quite general in nature. A much greater number of rules would be required to account for very subtle changes, such as the case for the disubstituted alkyl halide.

On the basis of the very limited data analysed, it appears that the (Q)SARs and the *in vitro* tests may be able to complement one another in a testing strategy, with the (Q)SAR/read-across information correctly identifying positives where the *in vitro* tests generate false negatives, and the *in vitro* tests correctly identifying negatives where the (Q)SARs generate false positives.

3.4 Application of the BfR rulebase for skin irritation

Background on the BfR Decision Support System (DSS), and an analysis where the BfR physicochemical and structural rules were applied to the 60 test chemicals of the validation study, can be found in Appendix 2.

With regard to the chemicals which have been misclassifed by the *in vitro* assays, the BfR DSS could generate predictions for only 4 out of the 21 non-confidential chemicals (i.e. chemicals 17, 34, 54 and 60). None of these chemicals were part of the BfR DSS training set. Out of the 4 chemicals, 2 EU non-irritant chemicals were correctly predicted with the BfR rule base (i.e., chemicals 17 and 54), and 2 EU R38 chemicals were misclassified also with the BfR DSS rulebase (i.e., chemicals 34 and 60). Consequently, the BfR DSS applies to relatively few of the chemicals that were misclassified *in vitro*, which does not provide a sufficient basis to draw strong conclusions.

4. REVIEW OF PHYSICAL-CHEMICAL PROPERTIES OF MISCLASSIFIED CHEMICALS

Twenty-one test chemicals, indicated as misclassified according to predictions from the *in vitro* skin models EPISKIN and Epiderm, have been reviewed in relation to known physical chemical properties, including octanol-water partition coefficient (Log K_{ow}), water solubility, vapour pressure, melting point, and boiling point.

Five chemicals, classified from *in vivo* data as R38 (GHS category 2 irritant) were variously mis-predicted as false negatives (Table 4.1).

Table 4.1. R38 (GHS category 2 irritant) chemicals misclassified as false negatives.

Chemical Nº.	**Decoding Nº.**	**CAS Nº**	**Chemical Name**
40	4	5271-27-2	1-methyl-3-phenyl-1-piperazine
3	9	111-25-1	1-bromohexane
23	11	629-19-6	di-n-propyl disulphide
18	12	97-88-1	butyl methacrylate
27	13	129228-21-3	2-isopropyl-2-isobutyl-1,3-dimethoxypropane

Four of these chemicals are liquids with low melting points, in the range -75 to <-100°C (i.e., significantly lower than the melting point of ice/water). Considering these low melting points, the chemicals might be volatile at room temperature, relative to water. By contrast, the vapour pressure of water (3168 Pa at 25°C) is six times higher than the highest vapour pressure value (520 Pa at 25°C) available for these five chemicals. However, the misclassified liquid chemicals also have high boiling points relative to water, in the range 155 to 202°C, i.e., exhibiting a liquid temperature range significantly wider than that of water. The high boiling points would be consistent with the relatively low vapour pressures. Nevertheless, to interpolate/extrapolate further would be limited by uncertainty in the phase relationships (and non-linearity of vapour pressure) of the substances with respect to temperature. However, in conclusion it might be expected that the liquid false negative R38 irritants could generate significant vapour, relative to water, at ambient temperature. For the five chemicals, water solubility ranges from 5 to 958000 mg/L, while Log K_{ow} ranges between 0.9 and 4.3.

Seven chemicals, classified from *in vivo* data as R38 (GHS category 3 mild irritant) were variously mis-predicted as false negatives, and a single chemical, classified from *in vivo* data as non-R38 (GHS category 3 mild irritant) was misclassified by all six participating laboratories as false positive (Table 4.2).

Table 4.2. R38 (GHS category 3 mild irritant) chemicals misclassified as false negatives and a non-R38 (GHS category 3 mild irritant) chemical (nº. 17) misclassified as false positive.

Chemical Nº.	**Decoding Nº.**	**CAS Nº**	**Chemical Name**
13	14	68047-07-4	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one
51	15	52783-21-8 (mix.)	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS# 191044-60-7) 1-(3-isopropylphenyl)-1-phenylethane (CAS# 191044-59-4) 1-(4-isopropylphenyl)-1-phenylethane (CAS# 2320-06-1)

34	17	6259-76-3	hexyl salicylate
55	18	80-26-2	terpinyl acetate
49	20	152848-22-1	isostearic acid monoisopropanolamide
60	24	not allocated	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex
43	25	not allocated (mix.)	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane (CAS# 181258-87-7) 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane (CAS# 181258-89-9)
17	30	128119-70-0	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer

The physical chemical properties of these eight chemicals show a range of values (where data available). However, similar to the previous group, four chemicals (51, 60, 43, 17: where data available) show a liquid temperature range significantly wider than that of water, with melting points in the range <-25 to <-50°C and boiling points in the range 241 to 309°C. Again, the high boiling points would be consistent with the low vapour pressures of the liquids at ambient temperature (<0.0001 to 44.5 Pa) but coupled to the relatively low melting points, the liquids might be expected to exhibit apparent volatility relative to water at ambient temperature.

Log K_{ow} values available are all positive, in the range 2.3 to 7, and tending toward the upper range typical of organic molecules, indicative of hydrophobic and/or lipophilic character. For one particular substance (60: zinc complex) dispersion in water has been reported to result in separation into three phases. Moreover, octanol-water partition coefficient data for the zinc complex are reported for three hydrolysis products. However, this chemical was reported false negative in only two laboratories (EPISKIN). For the eight chemicals in this group, water solubility (where data available) ranges from <1 ppm to 51000 mg/L.

Eight chemicals, classified from *in vivo* data as non-R38 (GHS no category non-irritants) were variously mis-predicted as false positives (Table 4.3).

Table 4.3. non-R38 (GHS no category non-irritant) chemicals misclassified as false positives.

Chemical Nº.	**Decoding Nº.**	**CAS Nº**	**Chemical Name**
54	34	not allocated (mix.)	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one (CAS# 224031-70-3) 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS# 224031-71-4)
35	38	2550-52-9	cyclohexadecanone
5	39	350-30-1	3-chloro-4-fluoronitrobenzene
8	42	3446-89-7	4-methylthio-benzaldehyde
6	49	5351-04-2	3-diethylaminopropionitrile
2	50	6940-78-9	1-bromo-4-chlorobutane
7	57	51755-83-0	3-mercaptohexanol
26	58	2820-37-3	3,4-dimethyl-1H-pyrazole

The physical chemical properties of these eight chemicals show a wide range of values. However, four of these chemicals were not selected from NCD and are reported with only scant availability of data on physical chemical properties. Two of the chemicals (35 and 7) are reported with wide liquid temperature ranges (i.e., melting points: <-50°C and -70°C; boiling points: 296°C and 245°C, respectively) with analogous implications already indicated above.

Where data are available for these eight chemicals, water solubility occurs in the range <1 to 65600 mg/L, and $LogK_{ow}$ covers the range 1.3 to 6.4.

5. EVALUATION OF A POTENTIAL RELATIONSHIP BETWEEN MISCLASSIFICATION OF CHEMICALS AND RISK PHRASES OTHER THAN R38

The objective of the present evaluation is to investigate whether chemicals that are non irritants but present skin sensitisation (risk phrase R43) or eye irritation (risk phrases R41 for severe irritants and R36 for irritants) effects could interact with the *in vitro* skin models in ways which may lead to their misclassification.

To evaluate this hypothesis, the relationship between R43, R41 and R36 risk phrases and the final correct or misclassifications were investigated for the 60 chemicals, in particular for the 34 non-R38 chemicals, tested in Phase II.

The evaluation has been performed by using:

1. The MTT results for correct or misclassifications provided by Sebastian Hoffmann.

2. The official classification & labelling (C&L) published in Annex I or the Competent Authorities proposals for C&L based on the *in vivo* responses indicated in the notification files.

3. Generally, for the commercially available chemicals, an official C&L was not available. In those cases, the C&L reported in the MSDS from the commercial suppliers were used. It is important to note that the sources for the commercial C&L provided by the chemical suppliers are not traceable and standardised as it is the case with the C&L published in Annex I or those proposed by the Competent Authorities.

In general, the number of chemicals labelled R36, R41 and R43 included in the set of tested chemicals selected, were too low to allow for statistical analysis with significant outcome. However, the general pattern was evaluated as described below.

5.1 Relationship between misclassification and risk phrase 43

The R43-labelled chemicals represented 9 out of the 60 tested chemicals, and 3 out of the 34 chemicals with no R38 label.

There was one R43, non-R38 labelled chemical (out of 3) misclassified *in vitro* as a false positive. This was the mixture of isomers:
1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one (CAS# 224031-70-3)
1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS# 224031-71-4).

The other two R43, non-R38 labelled chemicals were correctly classified *in vitro*. These were:
- *2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate*
- and *2-(formylamino)-3-thiophenecarboxylic acid*.

No clear pattern of a possible relation between R43 labelling and the *in vitro* misclassification for skin irritation could be observed.

5.2 Relationship between misclassification and risk phrase 41

The R41-labelled chemicals represented 7 chemicals out of the 60 tested chemicals, and 2 out of the 34 chemicals with no R38 label.

There was one R41, non-R38 labelled chemical (out of two) misclassified *in vitro* as a false positive: *3,4-dimethyl-1H-pyrazole*.

The other R41, non-R38 labelled chemical, *naphthalene acetic acid*, was correctly classified *in vitro*.

No clear pattern of a possible relation between R41 labelling and the *in vitro* misclassification for skin irritation could be observed.

5.3 Relationship between misclassification and risk phrase 36

The R36-labelled chemicals represented 15 chemicals out of the 60 tested chemicals, and 7 out of the 34 chemicals with no R38 label. Three out of the 7 R36, non-R38 labelled chemicals were misclassified *in vitro* as false positives. No clear pattern of a possible relation between R36 labelling and the *in vitro* misclassification for skin irritation could be observed.

5.4 Conclusions

In general, the results show no clear pattern that a non-R38 chemical labelled R36, R41 or R43 could be misclassified *in vitro* as a false positive. The chances of being misclassified are less or equal to one chance over two.

5.5 Remarks on the R38 classification

For the selection of chemicals used in the SIVS, the CSSC based primarily the selection criteria on the availability of the raw *in vivo* data from which the skin irritation classifications were deducted.

The deducted classifications did always correspond to those published in Annex I or to those proposed by the Competent Authorities. However, for the commercially available compounds, there were 8 cases where the classification deducted from the *in vivo* data of the ECETOC database differed from the information given in the MSDS by the commercial supplier of the test substance.

Due to the fact that the sources for the commercial C&L provided by the chemical suppliers are not traceable or standardised like the official classifications published in Annex I, the CSSC considered only the classification obtained from the ECETOC database *in vivo* raw data.

Out of the 26 skin irritants (R38) tested in Phase II, two commercially available compounds had no classification assigned in the MSDS provided by their supplier. Only one out of the two chemicals (*di-n-propyl disulphide*) was misclassified *in vitro* as a false negative. The other (*tri-isobutyl phosphate*) was correctly classified as skin irritant.

Out of the 34 non skin irritants (non-R38), 6 commercially available compounds were found to have a R38 label assigned in the MSDS provided by their supplier. Only two out of the six chemicals were misclassified *in vitro* as false positives.

Here again, there was no clear pattern of *in vitro* misclassification (less or equal to one chance over two) for the 8 commercially available chemicals that had a different C&L indicated by their suppliers in comparison to the skin irritation classification deducted from the raw *in vivo* scores (ECETOC database).

6. EVALUATION OF THE COMMENTS RECEIVED BY THE PARTICIPATING LABORATORIES

The present evaluation was based on the comments received from the participating laboratories at the following dates:

EPISKIN model	EpiDerm model
L'Oréal, 15 Dec 05	Zebet, 13 Jan 06
Unilever, 03 Jan 06	IIVS, 19 Dec 05
Sanofi, 13 Jan 06	BASF, 04 Jan 06

All laboratories reported on observed anomalies of test chemicals when compared to the chemical description provided. Additional comments were given by some laboratories, however they were documented in various formats and presented different contents amongst laboratories.

For the present evaluation, only comments with clear relevance for investigating possible reasons for misclassification were considered. These include for example anomalies of tested chemicals with regard to the expected chemical description or possible remarks regarding physico-chemical properties. The evaluation did not take into account any technical remarks such as interaction of the compound with the nylon mesh or decolouration of culture medium at this stage.

6.1 Relationship between laboratory comments and misclassification of chemicals

The number of chemicals where comments were reported for the EPISKIN and EpiDerm models and the relation with possible misclassification of these in at least one laboratory is shown in Tables 6.1 and 6.2 respectively. There were 5 misclassified chemicals for the EPISKIN model (out of 14), and 12 misclassified chemicals for the EpiDerm model (out of 18), where no comments were received from the EPISKIN and EpiDerm laboratories respectively.

Table 6.1. Relationship between the number of chemicals with comments from EPISKIN laboratories and misclassification of these in at least one laboratory. NQ= non-qualified.

Reporting laboratory	Total no. of chemicals with comments	Number of misclassified chemicals in		Number of correctly classified chemicals with comments	Chemicals misclassified within the reporting laboratory
		EPISKIN model (also in EpiDerm)	EpiDerm but not in EPISKIN		
L'Oréal	8	3 (1)	1	4	1 (+ 1 NQ)
Sanofi	5	3 (2)	1	1	3
Unilever	24	7 (4)	3	14	5

Table 6.2. Relation between the number of chemicals with comments from EpiDerm laboratories and misclassification of these in at least one laboratory.

Reporting laboratory	Total n. of chemicals with comments	Number of misclassified chemicals in		Number of correctly classified chemicals with comments	Chemicals misclassified within the reporting laboratory
		EpiDerm model (also in EPISKIN)	EPISKIN but not in EpiDerm		
Zebet	6	3 (1)	2	1	2
IIVS	6	4 (2)	2	0	3
BASF	3	0 (0)	1	1	0

6.2 Detailed comments and CSSC remarks

To search for insights on causes for misclassifications, the detailed comments received from the participating laboratories were evaluated on a case-by-case basis for those chemicals that presented concerns of misclassification. Table 6.3 shows the detailed comments received on chemicals misclassified in at least one laboratory and the CSSC evaluation and possible interpretation.

Table 6.3: Comments received on chemicals misclassified in at least one laboratory and CSSC observations and possible interpretation.

Decoding N°	Chemical Name (database source)	*In vivo* C&L	*In vitro* result	Comments from the laboratories	CSSC observations	CSSC Possible interpretation
4	1-methyl-3-phenyl-1-piperazine (NCD)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) F- (IIVS) OK (BASF)	**L'Oréal** Solid, crystalline powder, white to pale yellow that became soluble with water contact on the surface of the epidermis **Sanofi** Solid, crystalline powder that was found to be soluble with air contact. **IIVS** Non-cytotoxic in two first trials, where tissues had more edge aberrations and moisture on the surface. But results not so different between 3 tissues. Could compound be more toxic within time?	IIVS is the only lab where the chemical was misclassified. The compound has a very high water solubility (>/= 958000 mg/L)	Investigate whether tissues with edge aberrations used by IIVS could lead to misclassification.
9	1-bromohexane (ECETOC)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	**Unilever** Some variability in appearance seen after MTT with batch 050-EKIN-012, which reflected % viability and may reflect dosing differences **Zebet** Positive in large EpiDerm tissues EPI-606-X which has thicker SC. Hypothesis that chemical may not be washed so efficiently from thicker SC **IIVS** Is it possible that the material is highly volatile and not aqueous soluble such that in the 15 min exposure the relatively hydrated EpiDerm tissue, that the material volatised off rather than diffuse into the EpiDerm construct?	Misclassifications observed only in the EpiDerm model. No clear trend observed between single physico-chemical parameters and misclassification. The compound presents a vapour pressure 6 times smaller than water. However, it has a lower melting point and a higher boiling point as compared to water. The compound could generate more vapour than water at room temperature. It also has a positive Log Kow tending towards the upper range of values, and not too high water solubility.	Unclear. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.

12	Butyl methacrylate (TSCA)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) F- (IIVS) NQ (BASF)	**Zebet** Classified as Irritant on EPI - 606	Misclassifications observed only in the EpiDerm model. No clear trend observed between single physico-chemical parameters and misclassification. However, similar to chemical n. 9 the compound presents a lower melting point and a higher boiling point as compared to water. It also has a positive Log Kow tending towards the upper range of values, and not too high water solubility.	Unclear. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.
15	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS# 191044-60-7) 1-(3-isopropylphenyl)-1-phenylethane (CAS# 191044-59-4) 1-(4-isopropylphenyl)-1-phenylethane (CAS# 2320-06-1) (NCD)	EU R38 GHS mild irritant	F- (L'Oréal) F- (Unilever) F- (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	**IIVS** Suggest this compound as a good candidate for other toxicity endpoint	No other risk phrase than danger for the environment and aquatic organisms, and R38. *In vivo* data analysis showed possible *in vivo* misclassification.	Possible *in vivo* misclassification.
24	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex (NCD)	EU R38 GHS mild irritant	F- (L'Oréal) OK (Unilever) F- (Sanofi) OK (Zebet) OK (IIVS) OK (BASF)	**Unilever** On arrival the bottle was found to have leaked slightly. Material that possibly interacted with MTT but formed a turquoise colour rather than the normal blue/purple seen with direct reduction of MTT **IIVS** Residues could have increased toxic effects? **BASF** Rests (residue?) of test substance on skin: skin turquoise	*In vivo* data analysis showed possible *in vivo* misclassification. However, misclassification observed only with the EPISKIN model. MSDS recommends to avoid static electricity discharge. Could residues be due to handling conditions? Could turquoise colour affect classification?	Unclear.

30	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer (NCD)	EU non-R38 GHS mild irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) F+ (Zebet) F+ (IIVS) F+ (BASF)	**Unilever** When material used the seal inside the cap was found not fit properly (sucked into pot) and may have been affected by or interacted with the test material.	*In vivo* analysis showed possible *in vivo* misclassification.	Possible *in vivo* misclassification.
34	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one (CAS# 224031-70-3) 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS# 224031-71-4) (NCD)	EU non- R38 GHS non-irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) F+ (IIVS) OK (BASF)	**Unilever** Morphology after MTT showed irregular shaped blue/pink areas in all experiments suggesting dosing was not even.	Misclassifications observed only in the EpiDerm model. No clear trend observed between single physico-chemical parameters and misclassification. However, similar to chemical n. 9 the compound presents lower melting point and higher boiling point as compared to water. It also has a positive Log Kow tending towards the upper range of values, and low water solubility.	Unclear. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.
38	Cyclohexadecanone (NCD)	EU non- R38 GHS non-irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) OK(IIVS) F+ (BASF)	**Unilever** White specks were observed on tissues from batches 050-EKIN-016 and -018 after MTT assay – could be test material left on after washing (this was a liquid) or dead cells	BASF is the only laboratory where the chemical was misclassified.	Investigate whether any specific notifications not commented by BASF.
39	3-chloro-4-fluoronitrobenzene (ECETOC)	EU non-R38 GHS mild irritant	NQ (L'Oréal) F+ (Unilever) F+ retested (Sanofi) OK (Zebet) OK (IIVS) OK (BASF)	**L'Oréal** Expected to be whitely liquid but it was solid, crystalline powder **Unilever** Corrected sample by RCC-CCR **Zebet** Solid (crystals) not liquid **BASF** Solid not liquid	Solidification of samples could indicate test material decomposition. However, misclassifications observed only in the EPISKIN model.	Unclear.

42	4-methylthio-benzaldehyde (ECETOC)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ retested (Unilever) F+ (Sanofi) OK (Zebet) OK retested (IIVS) F+ (BASF)	**L'Oréal** Expected to be colourless liquid, but it precipitated and formed important deposit **Sanofi** Expected to be colourless liquid, but it precipitated and formed important deposit **Unilever** Material interacted with MTT	In contrast to the laboratory comments, chemical MSDS reports: "deep yellow green colour" MSDS also reports: "clear liquid".	Formation of important deposit could indicate test material decomposition.
49	3-diethylamino-propionitrile (ECETOC)	EU non-R38 GHS non-irritant	F+ retested (L'Oréal) OK (Unilever) NQ (Sanofi) OK retested (Zebet) NQ (IIVS) F+ (BASF)	**Unilever** Morphology after the MTT assay showed in general blue central areas and surrounding white areas on all tissues suggesting that dosing or effects were not even	Physico-chemical properties not retrieved for this commercially available compound.	Unclear. Further investigate physico-chemical properties and whether other laboratories also observed difficulties in dosing.
50	1-bromo-4-chlorobutane (ECETOC)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) OK retested (Zebet) NQ (IIVS) F+ (BASF)	**Zebet** Certain turbidity at the end. ZEBET comment: some precipitates occurred at the end of testing (no contamination) - maybe the sample was not stable anymore. Re-ordered sample was clear liquid and remains stable New substance from RCC tested once (PM 20 min/42h) - mean viability 49,5% ZEBET comment: similar structure as 1-bromohexane (R38), however classified *in vivo* as NI. Is the classification OK? **IIVS** Surprised it is not irritant such as 1-bromohexane as similar structure. How physico-chemical properties compare between both?	Classification based on ECETOC *in vivo* scores confirms the different scoring as compared to 1-bromo-hexane. Structure-activity relationships analysis indicates possibility that Br group leads to misclassification. However no misclassification observed with 1-bromohexane (chemical n. 9) on the EPISKIN model.	Unclear.

57	3-mercaptohexanol (NCD)	EU non-R38 GHS non-irritant	OK (L'Oréal) F+ (Unilever) F+ (Sanofi) NQ (Zebet) NQ (IIVS) NQ (BASF)	**L'Oréal** Expected to be clear odourless liquid but it was unclear (cloudy), colourless and with odour **Sanofi** Expected to be odourless liquid and it was found to be colourless with odour **Unilever** Expected to be clear liquid, but it was milky with strong odour. Strong interaction with MTT **Zebet** Turbid, not clear liquid. ZEBET comment: reaction with rubber leads to the turbidity of the sample and separation into the 2 phases. In the bottle with teflon lid no changes of the sample happen. Sample remained clear, in one phase. New substance from RCC was tested in all 3 runs. **IIVS** The cap seal of the test article primary container vial showed evidence of deformation and deterioration, presumably as a result of interactions with the test article. The progression of deterioration was observed temporally (sic) through the study. MTT reducer in killed control tissues with results that fell near the 50% cut-off with equivocal results.	Chemical MSDS reports: "characteristic odour" in contrast to the laboratory comments. It also reports: "clear and colourless liquid", and "Store in original container. Do not store in heat or direct sun light. Keep container tightly closed in a dry and well-ventilated place. Keep cool and away from light." Cloudiness of samples could indicate test material decomposition. Misclassifications observed in the EPISKIN model and results did not qualify in all labs evaluating EpiDerm assay.	Unclear.

58	3,4-dimethyl-1H-pyrazole (NCD)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) F+ (Zebet) F+ (IIVS) F+ (BASF)	**Sanofi** Expected to be yellow solid. Solid in tube was very hard, needed to be scratched before use. **Unilever** Large crystal that were ground to enable weighing and dosing. After the MTT assay the cultures were mainly white but with blue areas suggesting uneven dosing or test material effects.	MSDS describes product to be in the form of "flakes", and recommends to store only in original container.	Strong solidification could indicate possible test material decomposition and difficulties to obtain homogeneous dosing.

7. CONCLUSIONS

The concluding chapter summarises the possible causes for misclassification reported in the previous chapters based on the following evaluations:

- *In vivo* misclassification
- Single and combined set of physico-chemical characteristics
- Structure-activity relationships
- Interaction of other toxicological endpoints such as skin sensitisation and eye irritation
- Comments from all participating laboratories

In addition, possible concerns of test material stability as notified in the MSDS but not notified by the participating laboratories were included when considered of relevance.

In total, 21 chemicals were misclassified in at least one laboratory. Whereas some chemicals were misclassified in both EPISKIN and EpiDerm models, others were misclassified in only the EPISKIN or EpiDerm models. Tables 7.1-7.6 summarise the distribution of misclassifications with respect to skin model and participating laboratory. In the Tables, the following notation is adopted to describe *in vitro* result:
F- = false negative
F+ = false positive
NQ = non-qualified
OK = correct prediction

7.1 Chemicals misclassified in both EPISKIN and EpiDerm models

Table 7.1 Chemicals misclassified in the 6 participating laboratories.

Decoding N°.	Chemical Name (database source)	*In vivo* C&L	*In vitro* result	CSSC interpretation
15	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane 1-(3-isopropylphenyl)-1-phenylethane 1-(4-isopropylphenyl)-1-phenylethane (NCD)	EU R38 GHS mild irritant	F- (L'Oréal) F- (Unilever) F- (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	*In vivo* data analysis showed possible *in vivo* misclassification.
17	Hexyl salicylate (ECETOC)	EU R38 GHS mild irritant	F- (L'Oréal) F- (Unilever) F- (Sanofi) F- (Zebet) F- (IIVS) F- (BASF	Unknown
20	Isostearic acid monoisopropanolamide (NCD)	EU R38 GHS mild irritant	F- (L'Oréal) F- (Unilever) F- (Sanofi) F- (Zebet) F- (IIVS) F- (BASF	*In vivo* data analysis showed possible *in vivo* misclassification. In addition, this compound present structure-activity relationship that may allow for misclassification discrimination (presence of secondary amine group).

30	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer (NCD)	EU non-R38 GHS mild irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) F+ (Zebet) F+ (IIVS) F+ (BASF)	*In vivo* analysis showed possible *in vivo* misclassification.
58	3,4-dimethyl-1H-pyrazole (NCD)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) F+ (Zebet) F+ (IIVS) F+ (BASF)	Possible test material decomposition and difficulties of dosing. Laboratories commented on strong solidification and MSDS recommended to store only in original container. In addition, laboratory observation of uneven dosing. This compound also present structure-activity relationship that may allow for misclassification discrimination (presence of N in aromatic heterocycles).

Table 7.2 Chemicals misclassified in 4 out of 6 participating laboratories.

Decoding N°.	**Chemical Name (database source)**	***In vivo* C&L**	***In vitro* result**	**CSSC interpretation**
11	Di-n-propyl disulphide (ECETOC)	EU R38 GHS irritant	NQ (L'Oréal) OK (Unilever) F- (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	Unclear. No notifications from laboratories but MSDS recommendation to keep away from heat.
13	2-isopropyl-2-isobutyl-1,3-dimethoxypropane (NCD)	EU R38 GHS irritant	F- (L'Oréal) OK (Unilever) F- (Sanofi) F- (Zebet) OK (IIVS) F- (BASF)	Unclear. No notifications from laboratories but MSDS recommendation to store in polyethylene barrels or glass bottles.
42	4-methylthio-benzaldehyde (ECETOC)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) OK (Zebet) OK (IIVS) F+ (BASF)	Laboratories indicated formation of important deposit, which could indicate test material decomposition.

50	1-bromo-4-chlorobutane (ECETOC)	EU non-R38 GHS non-irritant	F+ (L'Oréal) F+ (Unilever) F+ (Sanofi) OK (Zebet) NQ (IIVS) F+ (BASF)	Unclear. Structure-activity relationships analysis indicates possibility that Br group leads to misclassification. However no misclassification observed with 1-bromohexane (chemical n. 9) on the EPISKIN model.

Table 7.3. Chemicals misclassified in 2 out of the 6 participating laboratories.

Decoding N°	**Chemical Name (database source)**	***In vivo* C&L**	***In vitro* result**	**CSSC interpretation**
18	Terpinyl acetate (ECETOC)	EU R38 GHS mild irritant	F- (L'Oréal) OK (Unilever) NQ (Sanofi) NQ (Zebet) OK (IIVS) F- (BASF	Unknown.
49	3-diethylaminopropionitrile (ECETOC)	EU non-R38 GHS non-irritant	F+ (L'Oréal) OK (Unilever) NQ (Sanofi) OK (Zebet) NQ (IIVS) F+ (BASF)	Unclear. Laboratory indicated an uneven dosing. The compound present structure-activity relationship that may allow for misclassification discrimination (presence of tertiary N-atom).

7.2 Chemicals misclassified in the EpiDerm model only

Table 7.4. Chemicals misclassified in 2 and 3 participating laboratories.

Decoding N°.	Chemical Name (database source)	*In vivo* C&L	*In vitro* result	CSSC interpretation
9	1-bromohexane (ECETOC)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	Unclear. No clear trend observed between single physico-chemical parameters and misclassification. The compound presents a vapour pressure 6 times smaller than water. However, it has a lower melting point and a higher boiling point as compared to water. The compound could generate more vapour than water at room temperature. It also has a positive Log Kow tending towards the upper range of values, and not too high water solubility. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.
12	Butyl methacrylate (TSCA)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) F- (IIVS) NQ (BASF)	Unclear. No clear trend observed between single physico-chemical parameters and misclassification. However, similar to chemical n. 9 the compound presents a lower melting point and a higher boiling point as compared to water. It also has a positive Log Kow tending towards the upper range of values, and not too high water solubility. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.

25	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane (NCD)	EU R38 GHS mild irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) F- (IIVS) F- (BASF)	Unclear. No clear trend observed between single physico-chemical parameters and misclassification. However, similar to chemical n. 9 the compound presents a lower melting point and a higher boiling point as compared to water. It also has a positive Log Kow tending towards the upper range of values, and not too high water solubility. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.

Table 7.5 Chemicals misclassified in 1 out of 3 participating laboratories.

Decoding N°.	**Chemical Name (database source)**	***In vivo* C&L**	***In vitro* result**	**CSSC interpretation**
4	1-methyl-3-phenyl-1-piperazine (NCD)	EU R38 GHS irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) F- (IIVS) OK (BASF)	Investigate whether tissues with edge aberrations used by IIVS could have led to misclassification
14	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one (NCD)	EU R38 GHS mild irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) F- (Zebet) NQ (IIVS) NQ (BASF)	Investigate whether any specific notifications not commented by Zebet.
34	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (NCD)	EU non-R38 GHS non-irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) F+ (IIVS) OK (BASF)	Unclear. No clear trend observed between single physico-chemical parameters and misclassification. However, similar to chemical n. 9 the compound presents lower melting point and higher boiling point as compared to water. It also has a positive Log Kow tending towards the upper range of values, and low water solubility. Further investigation recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.

38	Cyclohexadecanone (NCD)	EU non-R38 GHS non-irritant	OK (L'Oréal) OK (Unilever) OK (Sanofi) OK (Zebet) OK(IIVS) F+ (BASF)	Investigate whether any specific notifications not commented by BASF.

7.3 Chemicals misclassified in the EPISKIN model only

Table 7.6 Chemicals misclassified in 2 out of 3 participating laboratories.

Decoding N°.	**Chemical Name (source)**	***In vivo* C&L**	***In vitro* result**	**CSSC interpretation**
24	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex (NCD)	EU R38 GHS mild irritant	F- (L'Oréal) OK (Unilever) F- (Sanofi) OK (Zebet) OK (IIVS) OK (BASF)	Unclear. *In vivo* data analysis showed possible *in vivo* misclassification. However, misclassification observed only with the EPISKIN model. Laboratories reported the presence of residues and of turquoise coloration. MSDS recommends to avoid static electricity discharge. Could residues be due to handling conditions? Could turquoise colour affect classification?
39	3-chloro-4-fluoronitrobenzene (ECETOC)	EU non-R38 GHS mild irritant	NQ (L'Oréal) F+ (Unilever) F+ (Sanofi) OK (Zebet) OK (IIVS) OK (BASF)	Unclear. Solidification of samples notified by laboratories could indicate test material decomposition. However, misclassifications observed only in the EPISKIN model.

57	3-mercaptohexanol (NCD)	EU non-R38 GHS non-irritant	OK (L'Oréal) F+ (Unilever) F+ (Sanofi) NQ (Zebet) NQ (IIVS) NQ (BASF)	Unclear. Chemical MSDS recommends to "Store in original container. Do not store in heat or direct sun light. Keep container tightly closed in a dry and well-ventilated place. Keep cool and away from light." Cloudiness of samples indicated by laboratories could indicate test material decomposition. However, misclassifications observed only in the EPISKIN model.

7.4 Conclusions

Out of the 5 compounds misclassified in all 6 laboratories, in both EPISKIN and EpiDerm models:

- three could be compounds with possible *in vivo* misclassification,
- one shows indication of possible test material decomposition,
- Hexyl salicylate doesn't show any clear reasons for misclassification, but it is a GHS mild irritant, situated in the middle range of *in vivo* scores.
  Furthermore, salicylate being the salt of salicylic acid, it may have an anti-inflammatory action on the tissue.

Out of the 4 chemicals misclassified in 4 laboratories in both the EPISKIN and EpiDerm models:

- one show indication of possible test material decomposition,
- the three others show less clear possible reasons for misclassification.

For the four chemicals misclassified in 2 or 3 laboratories with the EpiDerm model only, no clear reasons were found for misclassification, however all compounds show a lower melting point and higher boiling point than water, as well a positive Log Kow tending towards the upper range of values, and low water solubility. Further investigation is recommended on possible tissue-specific incompatibility with a combined set of physico-chemical properties.

For the three chemicals misclassified in 2 laboratories with the EPISKIN model only, no clear reasons for misclassification were found. Further investigation is recommended.

Appendix 1

Correspondence of SIVS sequential chemical number (Phases I & II) with code numbers issued for reference of participating laboratories (EPISKIN & Epiderm) and decoding numbers assigned for impartial biostatistical analysis of results (ECVAM).

Final statistics report	EpiDerm				EPISKIN				Chemical identification	Epiderm/EPISKIN decoding
	ZEBET code		IIVS code	BASF code	L'Oréal code		Unilever code	Sanofi code		
	Phase I	Phase II	Phase II	Phase II	Phase I	Phase II	Phase II	Phase II		
1	42	163	103	759	85	403	135	740	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	7
2		578	113	527		355	569	493	1-bromo-4-chlorobutane	50
3		808	933	179		288	280	688	1-bromohexane	9
4		187	789	213		367	505	565	1-decanol	6
5		969	190	745		746	467	161	3-chloro-4-fluoronitrobenzene	39
6		630	315	671		300	948	342	3-diethylaminopropionitrile	49
7		455	867	225		149	952	605	3-mercaptohexanol	57
8		254	110	586		567	929	183	4-methylthio-benzaldehyde	42
9	72	207	115	546	43	125	985	526	2,6-dimethyl-4-nitrobenzeneamine	32
10		936	656	258		773	143	496	allyl heptanoate	28
11		541	160	686		572	427	814	allyl phenoxyacetate	41
12	46	747	249	347	81	636	375	949	2-ethylhexyl 4-aminobenzoate	46
13	33	864	915	989	19	818	299	974	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	14
15		735	337	900		204	134	691	a-terpineol	10
16	73	975	262	487	18	137	267	518	capryl-isostearate	47
17	87	966	239	622	63	779	261	902	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	30
18		501	334	633		782	813	973	butyl methacrylate	12
19		384	755	169		768	543	147	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	56
20		462	431	295		836	844	368	cyclamen aldehyde	5
21		570	480	805		682	608	616	A mixture of:	31
22		319	714	343		346	822	371	diethyl phthalate	51
23		215	363	780		658	673	330	di-n-propyl disulphide	11
24		593	222	547		799	920	404	di-propylene glycol	52
25		353	488	913		699	875	446	dipropylene glycol monobutyl ether	54
26		628	430	639		189	159	549	3,4-dimethyl-1H-pyrazole	58
27		503	109	184		716	833	170	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	13
28	95	581	713	697	30	266	447	889	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-	36
29	67	613	148	562	16	495	732	642	Mixture of:	16
30	20	750	519	287	90	341	855	558	Mixture of:	45
31		595	659	848		255	236	154	A mixture of isomers: ethyl exo-tricyclo[5.2.1.0(2,6)]decane-endo-2-carboxylate; ethyl endo-tricyclo[5.2.1.0(2,6)]decane-exo-2-carboxylate	21
32	21	762	528	162	40	139	827	662	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-	33
33		282	600	188		201	416	398	heptyl butyrate	29
34		276	232	719		815	684	726	hexyl salicylate	17
35	49	537	906	466	36	752	908	233	cyclohexadecanone	38
36		977	706	953		893	877	701	isopropanol	53
37	99	338	972	425	71	144	866	598	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl	2
39		794	723	722		379	326	817	methyl stearate	48
40	57	849	291	988	56	168	707	696	1-methyl-3-phenyl-1-piperazine	4
41		477	876	133		538	241	934	naphthalene acetic acid	55
42	89	890	409	859	66	298	824	323	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disul	37
43		345	471	542		399	756	865	A mixture of isomers:	25
44		535	152	927		274	583	606	phenylethylalcohol	40
45		971	676	821		269	680	959	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-	8
46		981	885	252		308	914	270	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]decan-2-ol	22
47		521	278	385		359	997	238	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	23
48		202	788	694		724	122	708	2-(formylamino)-3-thiophenecarboxylic acid	59
49	78	894	838	185	88	485	718	151	isostearic acid monoisopropanolamide	20
50		585	271	965		961	111	580	2-phenylhexanenitrile	27
51	29	947	872	126	65	568	121	223	Mixture of isomers:	15
52	35	648	445	235	53	883	164	366	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	35
53		459	158	832		197	123	856	silane A-1430	43
54		903	208	807		982	828	638	Mixture of isomers:	34
55		637	968	286		800	625	400	terpinyl acetate	18
56		120	141	476		461	321	247	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name*	3
57		576	124	200		964	992	539	triethylene glycol	44
58		797	917	749		743	655	456	tri-isobutyl phosphate	19
59	55	106	481	588	34	830	874	439	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	1
60		666	356	132		880	119	820	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	24

Appendix 2

Application of the BfR rulebase for skin irritation to the test chemicals in the ECVAM skin irritation validation study