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FOREWORD

This document presents the OECD Explanatory Background Document (EBD) supporting the *In Vitro* Skin Irritation Test Guideline No. 439. The OECD EBD is based on the joint explanatory background document of the European Centre for the Validation of Alternative Methods (ECVAM) at the European Commission's Joint Research Centre and the Centre for the Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment (BfR).

The purpose of the joint ECVAM/BfR document was to support the regulatory acceptance process in the EU and, in particular at OECD level. To this end the document describes the development, optimisation and validation of the three individual *in vitro* skin irritation test methods, which are now included into the Test Guideline No. 439, and provides in-depth background to the scientific foundations of the test methods, their applicability and their performance under the UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Moreover, the project for developing an OECD version of the joint ECVAM/BfR document was supported by an OECD Expert Consultation Meeting on Skin Irritation in November 2008. Comments provided by the *Ad Hoc* Skin Irritation Expert Group, led subsequently to a final draft which was submitted to the WNT for comments in December 2009. The EBD was approved by the WNT22 at its meeting held on 23-25 March 2010. The Joint Meeting of Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 15 September 2010.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

PREAMBLE

The development of this Explanatory Background Document (EBD) in support of the draft *In Vitro* Skin Irritation Test Guideline No. 439 was initiated in spring 2009 by ECVAM/BfR and a first draft of the EBD was discussed by an OECD Expert Consultation Meeting in June 2009.

The purpose for developing this EBD was as a response to a number of difficult comments raised in the earlier commenting rounds to the WNT. The history behind the development of the three individual *in vitro* skin irritation test methods, that are now included into the draft Test Guideline No. 439 is complex and encompasses several different validation studies and optimisation phases and the EBD clearly describes the most important steps in the development process, in addition to list all pertinent documentation. In addition to the development of Performance Standards for me-too test method development, the final test method also needed to be adapted and evaluated for the changed regulatory requirements, from the previous EU system (EU DSD) to the UN GHS. The EBD is a comprehensive review addressing all basic issues raised in the WNT commenting rounds, and in that respect it has served its purpose well.

A first draft EBD was developed by the European Centre for the Validation of Alternative Methods (ECVAM) of the European Commission and the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment (BfR). Comments have been provided by the Ad Hoc Skin Irritation Expert Group, before a final draft was submitted to the WNT for comments in December 2009. The EBD was approved by the WNT22 at its meeting held on 23-25 March 2010.

Many experts have participated in the OECD *In Vitro* Skin Irritation Project and the Secretariat would especially like to mention the experts that have authored this document:

– João Barroso EC, JRC/IHCP, Ispra, Italy
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The OECD Secretariat gratefully acknowledges these experts and the members of the *Ad Hoc* Skin Irritation Expert Group for their professional assistance and their indispensible contributions to the finalisation of this Explanatory Background Document and the draft In Vitro Skin Irritation Test Guideline No. 439.

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ABSTRACT

Since 1946 the Draize rabbit test for dermal irritation has been successfully used to evaluate the skin irritation potential of xenobiotics. Despite some advantages, the test has also a number of drawbacks (e.g. high inter-laboratory and inter-animal variability) that warrant the use of an alternative empirical testing method. Intense research by academia, industry and within publicly-funded research programmes (e.g. EU framework programme 4, FP4) has led to the development of *in vitro* tissue constructs based on human keratinocytes. These constructs closely resemble human epidermis with respect to biochemical profile (e.g. lipid composition), tissue architecture (e.g. cell layering and formation of a *stratum corneum*) and the presence of a functional skin barrier.

Three commercially available test methods based on this principle of "Reconstructed human Epidermis" (RhE) have been validated by the European Centre for the Validation of Alternative Methods (ECVAM) and found applicable for the determination of the presence and absence of hazardous (skin irritant) properties of xenobiotics also with respect to the new UN GHS-compliant rules for classification and labelling as implemented in the EU through the regulation on the Classification, Labelling and Packaging of Substances and Mixtures (CLP regulation 1282/200/EC, in force since 20 January 2009).

Since these three test methods are based on an identical tissue engineering technology (RhE) and use essentially the same test protocol (as evident from their associated Standard Operating Procedures, SOPs) they are amenable to the development of a generalised test method procedure, including minimal performance criteria for similar and modified methods. The need for an internationally harmonised and agreed test procedure (i.e. OECD test guideline, EU test method) for *in vitro* skin irritation testing based on RhE follows from the legislative requirements in some global areas (e.g. REACH and the Cosmetics Directive within the EU). Moreover, an internationally agreed test procedure is desirable to ensure a level playing field in the global arena with respect to the international acceptance of safety testing data concerning chemical, cosmetic, pharmaceutical substances and finished products and obvious benefits for the international commerce of such substances and products (e.g. avoidance of duplication of testing for different regional needs, resulting cost efficiency, non-ambiguity of test results due to high standardisation).

Scientifically, the development of internationally agreed test procedures is supported by the following considerations: (1) the test methods are based on the objective empirical measurement of cell/tissue damage, the initial key event triggering the inflammatory cascade leading to a localised dermal irritant response *in situ*, which underpins the method's mechanistic relevance and has implications on their chemical applicability domain. (2) the test methods have been assessed by rigorous empirical testing (i.e. validation) and were found to be highly reliable and relevant (also under UN GHS) with regard to predictions made by the reference *in vivo* method; (3) the test methods are based on human cells and may thus be of higher relevance in man.

Taking these factors into account, an *in vitro* skin irritation test method based on RhE technology has been drafted for inclusion within the EU test method regulation (Regulation 440/2008/EC) and for purposes of an OECD draft test guideline. The EU test method ("B.46") has received a positive recommendation by the EU committee on REACH and Classification and Labelling (so-called "CARACAL"); final adoption by the European Parliament is expected by summer 2009. The OECD draft Test Guideline on *in vitro* skin

irritation testing has undergone extensive commenting by OECD member countries and has been reviewed, considering with all relevant scientific aspects, in two OECD Expert Consultation Meetings (ECMs) in Berlin in October 2008 and Washington in June 2009. The initial version of this background document was intended to support the 2nd ECM in Washington. The present updated version takes the results and recommendations of this 2nd meeting into account. The document provides an executive summary that, in a concise manner, addresses all relevant aspects of the RhE technology, the scientific basis, the validation studies, applicability under UN GHS and the new GHS-compliant Performance Standards. All these points are elaborated in more detail in the remaining sections of the document. Test Guideline No. 439 on "*In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method*" was adopted by OECD Counsil on 22 July 2010.

EXECUTIVE SUMMARY

Purpose of the document

This document supports the ongoing expert consultations concerning the OECD draft Test Guideline (TG) on an *in vitro* test method for *in vitro* skin irritation testing based on reconstructed human epidermis (RhE), specifically in the context of the 2nd OECD Expert Consultation Meeting to be hosted by US EPA in Washington DC, USA 15-17 June 2009[1]. It moreover may support scientific discussions regarding EU test methods B.46 (in vitro skin irritation) of different EU expert committees charged with the questions pertaining to the use of test methods for safety assessments of xenobiotics (e.g. National/EU coordinators on test methods, the CARACAL committee and the ESAC).

This document is not intended to substitute the extensive background material (listed in Annex 1) which relates to the ECVAM validation studies and which was uploaded on the OECD protected Website in autumn 2008 for the 1st Expert Consultation Meeting on Skin Irritation that was hosted by BfR in Berlin, Germany. References to these documents are made with respect to their number in Annex 1 (*e.g. Annex 1 doc17 = Document Nr. 17 of the list provided in Annex 1*). The present background document may thus serve as a handbook from which more detailed inquires into the detailed publications and reports pertaining to the test methods can begin.

The main intention of this paper is (a) to bundle in a concise manner the essential information in one single document and (b) to provide background information on recent developments already discussed during the Berlin expert meeting and further extended here. These include the performance of the validated methods under UN GHS and the update of the ECVAM Skin Irritation Performance Standards according to UN GHS. The latter critically rest on discussions and agreements reached during the first OECD expert meeting in Berlin (e.g. Reference Chemicals, Target Accuracy Values).

0.1 Basis of the current Draft OECD TG: three ECVAM-validated test methods

0.1.1 The three test methods: EpiSkin, modified EpiDerm, SkinEthic

The present Draft OECD Test Guideline (Annex 1, doc28) as well as the EU test method B.46 (which is already well advanced with respect to the acceptance process by EU regulatory authorities), are primarily based on the evidence generated in two sets of validation studies on three commercially available RhE assays independently evaluated by ECVAM and peer reviewed by ECVAM's Scientific Advisory Committee (ESAC). These three assays are

(1) the EpiSkin™ test method;
(2) the modified EpiDerm™ test method[2] and
(3) the SkinEthic™ test method.

[1] This document has been updated following mentioned OECD expert consultation meeting and the recommendations and endorsements reached during this meeting have been embedded in the appropriate sections of this document and are provided in synopsis in Annexe 7.

[2] The EpiDerm test method underwent full prospective validation during the ECVAM Skin Irritation Validation Study (SIVS) on the basis of its original protocol featuring 15 minutes exposure time ("original EpiDerm"). Upon completion of the SIVS in 2007, the original EpiDerm test method was found to be useful of identification of positives (i.e. for use in a test strategy). The ESAC recommended a modification of the protocol. In spring 2008 a modified protocol of the EpiDerm assay with a prolonged exposure time of 60 minutes was submitted to ECVAM. In agreement with the provisions of OECD guidance document Nr. 34 on the validation of test methods, this modified EpiDerm assay was validated in November 2008 on the basis of the original ECVAM skin irritation performance standards.

All three have been found scientifically valid for reliably predicting no label and R38 (irritant) substances in respect to the previous EU classification scheme and have been confirmed in April 2009 by ESAC for use under the UN GHS system as *"applicable to all authorities"* (United Nations 2008).

0.1.2 Principal elements of the test methods

All three test methods use essentially the same protocol and to feature the same essential components (Figure 0.1), i.e. (1) test system, (2) endpoint, (3) prediction model which are used in a specific way as described in the test procedure (4) and associated Standard Operating Procedure (SOP).

The test system (1) employed is reconstructed human skin based on human-derived keratinocytes. While the test systems employed by the methods are not identical (e.g source of keratinocytes, growth conditions), the test systems are essentially and sufficiently similar, insofar as they reproduce human epidermis with the characteristic biochemical profile (e.g. lipid expression), the characteristic tissue architecture (e.g. layering of keratinocytes, cell morphology) and the formation of a stratum corneum. While differences between the test systems may impact for instance on the quantitative aspects of parameters (e.g. the barrier function of the skin models as measured through time to toxicity (ET_{50}) values for benchmark substances and controls vary between the three test methods), the test systems employed are qualitatively the same.

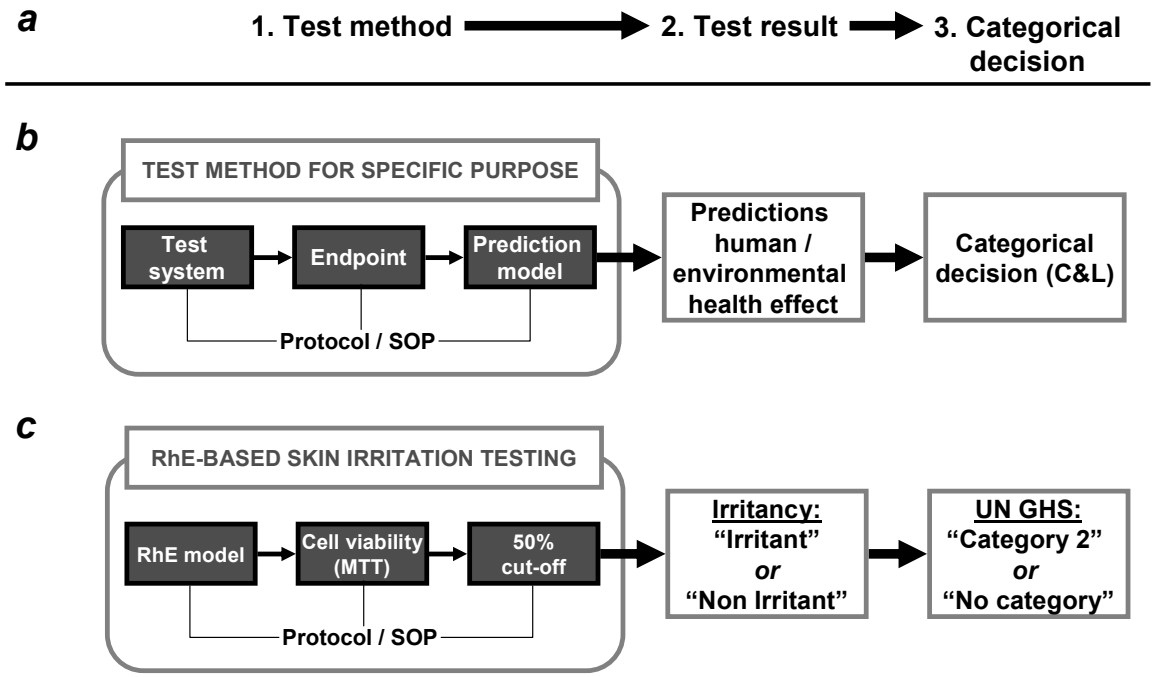
The endpoint (2) measured is in all three methods cell viability which is assessed through the reduction of the vital dye MTT to the blue formazane reaction product.

The prediction model (3) for translating the parameter measured *in vitro* (e.g. optical density of the MTT formazane reaction product normalised to control tissues) into a prediction of the adverse effect of the xenobiotic in the species of interest (humans) is identical for all three models: a simple cut-off value (50% viability normalised to the control).

The ***test procedures (4)*** of the three assays differ essentially only with respect to the exposure (contact) time of the chemical applied to the surface of the reconstructed epidermis. The exposure times are (in minutes): Episkin: 15, Skinethic: 42 and modified Epiderm: 60. The exposure times are understood to reflect the different barrier properties of the test systems (i.e. the *specific* reconstructed epidermis used) and are adjusted for each test system in order to guarantee a dynamic response: the exposure time needs to be long enough to allow the development of measurable effects while being short enough to ensure that the system is not driven into saturation. All three models use, after rinsing, a post-incubation period of 42 hours. This period is a result of a test procedure optimisation process to achieve the best separation of irritant from non-irritants chemicals using a PM with a 50% viability cut-off.

Since the protocols are otherwise near-identical, they are amenable to the development of a generalised test method or test guideline, i.e. the current draft OECD TG and the EU test method B.46.

Figure 0.1: Schematic depiction of the key elements of a test method for toxicity testing. (a) *A test method is a standardized method (1) for generating test results on xenobiotics (2) relevant for effects on human health or the environment (2). Such results may be translated in categorical decisions (3) for the purposes of classification and labelling of xenobiotics.* ***(b)*** *Test methods are composed of three elements: test system (e.g. a specific mouse strain; a specific cell line), endpoint (e.g. barrier function of an epithelium) and prediction mode (PM). The PM is used to translate the measured or observed effects into predictive test results (e.g. genotoxic or non-genotoxic) on the basis of which specific categorical decisions may be taken. The use of these three elements is described in the test method protocol and associated Standard Operating Procedure (SOP).* ***(c)*** *In the case of RhE-based in vitro skin irritation tests (EpiSkin, EpiDerm, SkinEthic), the test system is reconstructed human epidermis (RhE), the endpoint is viability (measured through reduction of MTT), the prediction model a 50% cut-off value of cell viability normalised (expressed in %) to concomitantly run negative controls. The test results are non-irritant and irritant and categorical decisions are – under UN GHS – category 2 (irritant) or no-category.*



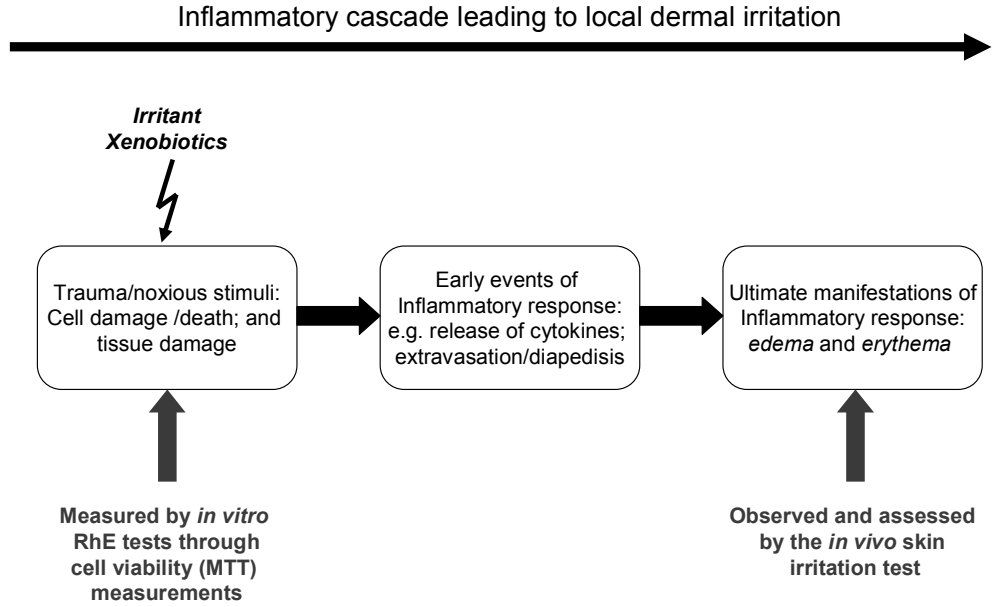
0.2 Scientific background

0.2.1 The in vivo Draize irritation test in rabbits: reference point for the in vitro methods

The development, optimisation and validation of the RhE in vitro skin irritation method was performed in reference to the Draize *in vivo* test method using albino rabbits. This test has been used for about half a century since its introduction in 1944 (Draize et al. 1944) although several improvements with regard to its protocol and the conclusions drawn have been introduced over time (adoption as OECD TG in 1981 with revisions in 1992 and 2002). The Draize method has the advantage of providing a simple readout of the downstream ("endpoint") effects of the inflammatory response produced in reaction to the tissue trauma/noxious stimuli induced by irritant xenobiotics. Such localised cell and tissue damage leads to release of inflammatory mediators, nerve stimulation, axonal reflexes, pain and itching (Wells et al. 2004; Kind et al. 2006; Fluhr et al. 2008). The inflammatory response ultimately leads to observable phenomena such as localised skin swelling (*edema* - due to increased permeability of blood vessels to facilitate

diapedesis/extravasation of immune cells into the interstitium) and redness (*erythema* - due to the increased diameter of blood vessels). These downstream events are visually observed and scored in the *in vivo* Draize test. Other reactions (e.g. C fibre activation, pain) are not easily accessible through such an observational method. While the animal test hence uses the ultimate manifestations of the irritancy cascade to make predictions on the irritancy potential of xenobiotics, the *in vitro* RhE methodology measures the primary event leading to the these ultimate manifestations of an irritant response: cell damage/death and, as a consequence, localised trauma (tissue damage) of the skin exposed (Wells et al. 2004). For a schematic drawing see Figure 0.2.1.

Figure 0.2.1 Schematic representation of the inflammatory cascade leading to local acute dermal irritation. *Xenobiotics, if irritant, induce localised cell damage/death and tissue damage in the epidermis/dermis exposed. This early event in the "irritation cascade" is measured by the in vitro methods through assessment of cell viability (MTT reduction). Localised trauma triggers an inflammatory response, including the release of inflammatory mediators (e.g. interleukins) as well as the increase of blood vessel permeability and diameter to expedite a localised response (e.g. extravasation of immune cells into the interstitium of the affected tissue. Thus the inflammatory response ultimately leads to the swelling and redness of the affected skin area.*



The *in vivo* method has however also several drawbacks:

(a) it is an ***animal test*** and as such increasingly questioned especially when used for consumer products that are not 'vital', i.e. that have no beneficial effects on human health (e.g. cosmetic ingredients);

(b) Coloured substances are difficult to score due the visual inspection of the skin and skin redness being a readout;

(c) Being performed in a proxy model (the rabbit) the test may make incorrect predictions due to ***species differences*** (e.g. Philips et al. 1972; Basketter et al. 2004).

(d) Probably due to the fact that the test relies on the subjective scoring of the two effects (instead of using an empirical measure of a parameter related to irritation) ***variability*** of the recorded responses is high (e.g. Weil and Scala 1971; ECETOC 1995);

(e) This variability may be exacerbated due to ***inter-individual (inter-animal) variability*** with regard to the severity of the responses.

The most systematic analysis of variability of Draize test data was performed already in 1971, when Weil and Scala (Weil and Scala 1971) distributed 10 substances to 24 laboratories and assessed intra- and inter-laboratory variability. The study found moderate within laboratory reproducibility and low between laboratory reproducibility and concluded that some of the substantial variability observed may be due to (1) the subjective way of scoring effects and (2) variations between laboratories in performing the test. However, high variability is also evident in the ECETOC database of skin irritation chemicals. Since these data were are all produced following OECD test guideline 404 and under Good Laboratory Practice (GLP), variations due to between-laboratory deviations in the test protocols can be excluded with high certainty. Hence, the variability is most likely based on either a) the subjective scoring or b) the intrinsic variability of responses in animals or both factors.

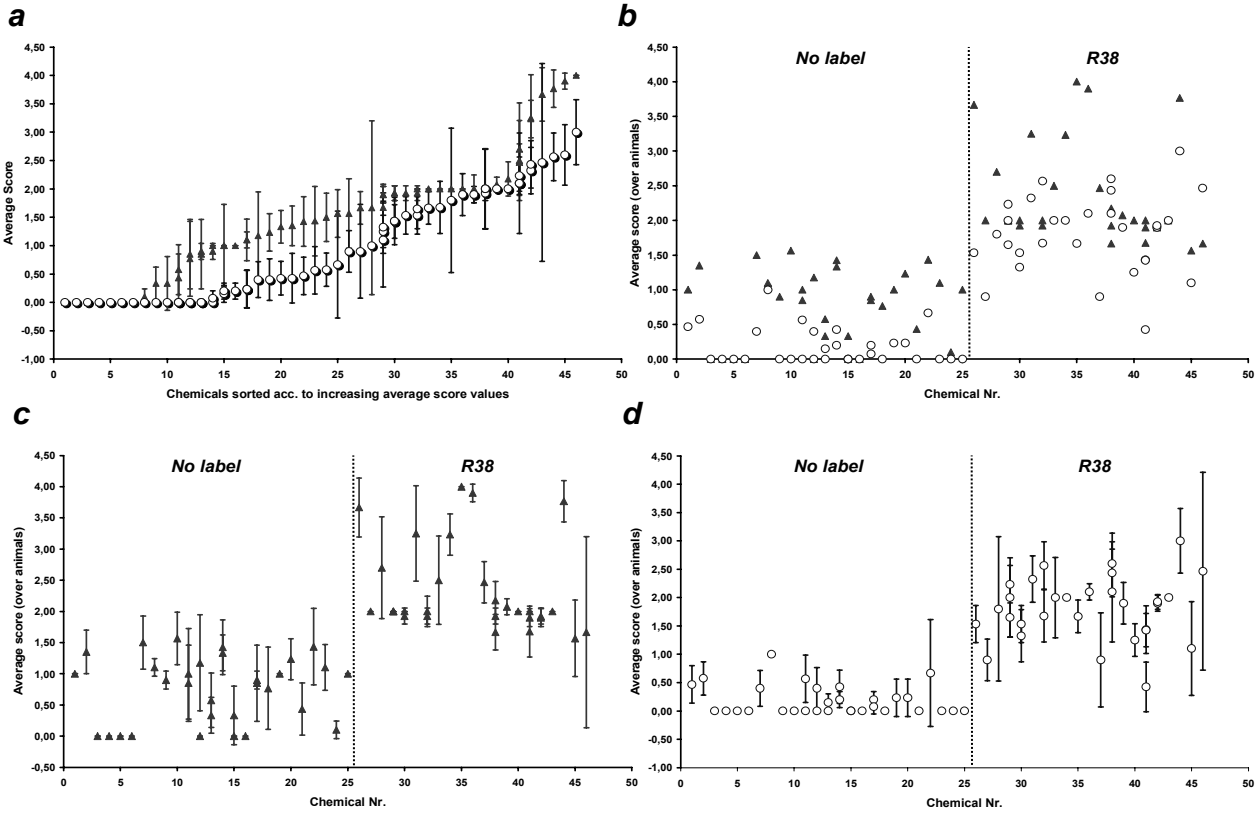
It is noteworthy in this context that one of the reasons for employing primarily one irritant category under UN GHS (category 2; category 3 is an optional opt-in) is inter-animal variability. The UN GHS text explicitly acknowledges that *"...animal responses in a test may be quite variable"* in the context of explaining the rationale for one single irritant category (category 2) (United Nations 2008; paragraph 3.2.5.1, sub point c).

Inter-animal (within test) and between test (laboratory) variability is also evident from the high quality dataset of the ECETOC skin irritation chemicals (ECETOC 1995). Figure 0.2.2 shows the erythema and edema scores of 45 chemicals of this set covering the entire range of irritancy Draize scores from 0 to 4.0. This data show high inter-animal variability, and it is, on the basis of these quality assured data, at least not immediately evident how the traditional *in vivo* test can indeed be used for sub-categorisation beyond one irritant category.

Consequently, a principal reason for optimising the *in vitro* models for discerning only one irritant category are the respective limitations of the *in vivo* test that served as reference point during optimisation and validation as the overwhelming majority of reference data are from Draize rabbit test data and C&L conclusions drawn on the basis of these data. Notwithstanding this fact, the capacity of the in vitro tests for possibly discerning two irritant categories (thus in total 3 logical categories including non-irritant/no label/no category) was assessed during the chemicals selection process of the ECVAM skin irritation validation study (see chapter 2).

Figure 0.2.2 Erythema (red triangles) and Edema (blue circles) irritancy scores of 45 chemicals from the ECETOC skin irritancy dataset produced in agreement with OECD TG 404 and under GLP. (a) *Averages of animal scores (± SD) of test results. Note that for some chemicals, more than one test is reported. The chemicals have been sorted according to increasing irritancy score.* ***(b-c)*** *Scatter plots of the averages of animal scores with chemicals plotted according to the number attributed in the ECETOC report. Note that for some chemicals there is more than one test report. Note the considerable variability in*

particular of edema test results (circles) for irritant (R38) chemicals and the large distribution of scores within this category. The stippled line separates no-label from R38 substances. Average erythema and edema scores without SD ***(b)****, average erythema* ***(c)*** *and edema* ***(d)*** *scores across animals (± SD). The data do support a dichotomous categorisation system (i.e. one irritant category, one non-irritant category) but further sub-categorisation on the basis of these data is not evident.*



0.2.2 Scientific work leading to the validated skin irritation in vitro methodology

The scientific work that led to the validation of these three methods as possible stand-alone methods for predicting irritant and as well as non-irritant chemicals covers in total about 30 years of research in the development and application of reconstructed human epidermis for safety assessments. Key events of this process have been outlined briefly below. With regard to the research activities that were already targeted at the possible future validation and regulatory use of the RhE methodology, the relevant period covers about 15 years of intensive research, optimisation and validation in industry and within EU-funded projects (e.g. the European Union's fourth framework programme for research from 1994 to 1998).

1. Up to the mid 1980ies: development of the ***basic methodology*** of reconstructing human epidermis in vitro (e.g. Pruniéras et al. 1983).
2. Mid 1990ies: A project conducted within the context of the ***EU Framework Programme 4 for Research*** (from 1994 – 1998) assessed possible parameters (endpoints) for predicting skin

irritation in human skin models. The project concluded that both MTT (cell viability) as well as the inflammatory mediator Interleukin-1 alpha were promising endpoints. However, due to the higher variability of IL-1 alpha, MTT was determined as the best endpoint for human skin models.

3. 1998: ***ECVAM task force on skin irritation*** published a report on the status of skin irritation testing and recommends 10 challenge chemicals for the assessment of test methods in the planned ECVAM prevalidation study (Annexe 1, doc 15).

4. 1998 – 2000***: ECVAM prevalidation study*** evaluating – amongst other tests – the readiness of the EpiSkin and EpiDerm test systems and their associated protocols (e.g. endpoint measured) and prediction models for entering full validation. Both were found reliable (i.e. reproducible within and between laboratories) but performed unsatisfactory in predicting the skin irritation potential of the 20 test substances. ECVAM recommended further test optimisation (See Annexe 1 doc17; doc18). The results of the prevalidation study are not relevant in this context and are not further elaborated in the present document.

5. 2001: ECVAM's skin irritation task force recommended developing ***a 'common protocol' for the in vitro reconstructed tissue models EpiSkin and EpiDerm*** before the start of a formal validation study.

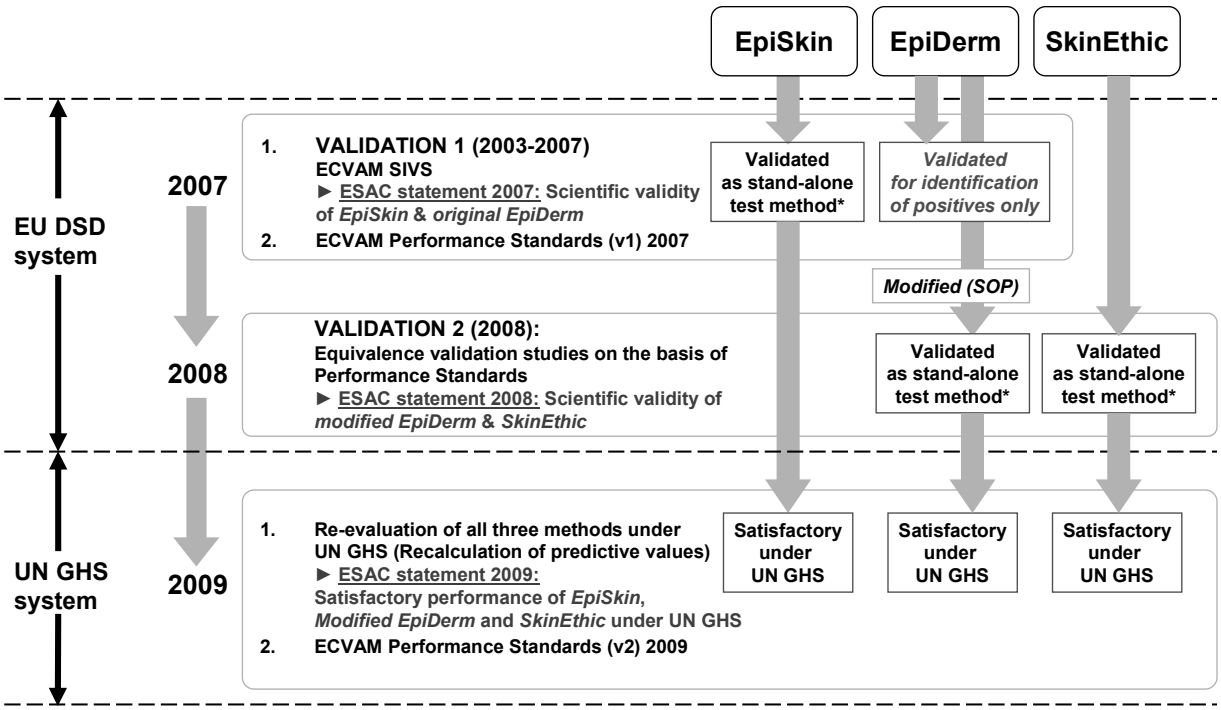
6. 2002: A comprehensive industry study showed ***good correlation between effects in man and the predictions made by reconstructed human epidermis models*** (among those the EpiSkin and the EpiDerm assay), underlining the relevance of the *in vitro* methods to the species of interest (Annexe 1, doc16).

7. 2003: Finalisation of ***two optimisation studies*** (published in 2005) pertaining to the EpiDerm and the EpiSkin test method (Annexe 1 doc19 and doc20). As a result of these studies, the common protocol (see point 5.) featured a 15 minute contact period with the test substance followed (after rinsing) by a 42 post-incubation period before cell viability was assessed. This protocol later (after the SIVS) turned out to be unsuitable for the EpiDerm test system, resulting in a low sensitivity.
8. 2003-2007: The ***ECVAM Skin Irritation Validation study (SIVS)***. Upon completion of the SIVS (Annexe 1 doc24, doc26), the EpiSkin test method using the MTT endpoint was found to be a reliable and relevant stand-alone method for distinguishing no label from R38 (irritant substances). At the time, the original EpiDerm method (which has been modified in the meantime) was recommended for the identification of positives within a testing strategy (ESAC statement from April 2007, Annexe 2).
9. The ***modified EpiDerm and the SkinEthic test methods*** were subsequently validated on the basis of these Performance Standards using the 20 defined Reference Chemicals (ESAC statement from November 2008, Annexe 3).

0.3 Validation key events leading to the draft OECD TG

With regard to the validation process of the three methods underlying the current draft OECD TG, it is useful to discern the following three key events marked by the publication of three consecutive ESAC statements from 2007 to 2009 (ESAC 2007 Annexe 2, ESAC 2008 Annexe 3, ESAC 2009 Annexe 4). These events are summarised in Figure 0.3:

Figure 0.3: Schematic representation of the major validation events. *These led to the drafting of the EU B46 test method and the OECD draft TG currently under discussion. Three major events can be discerned regarding the validation of the in vitro tests validated as potential full replacement methods (EU DSD system and UN GHS system as applicable to all authorities).*



**) as stand alone method for classification and labelling using one irritant category, i.e. as foreseen by the previous EU DSD system and the current UN GHS system as applicable to all authorities and implemented by the CLP regulation in the EU.*

Key events:

1) ***2007 - Validation of the EpiSkin and original Epiderm assays:*** The ECVAM Skin Irritation Validation Study (SIVS) was conducted from 2003 to 2007, financed by the European Commission (ECVAM) which also performed the independent statistical analysis of the data generated during the study. The SIVS was a prospective validation study involving the blind testing of 58 test substances representing a wide spectrum of chemical functionalities and representing the full range of dermal irritancy (Annexe 1 doc01 - doc08; doc14; doc26). The study featured two phases: Phase I during which the EpiDerm, EpiSkin and the skin integrity function test (SIFT) were evaluated (Annexe 1 doc10 – doc 13). Only the EpiDerm and the EpiSkin proceeded to Phase 2. Importantly, the study was designed also in view of the upcoming UN GHS system, e.g. with respect to the selection of test substances (Annexe 1 doc27; doc21). Moreover, not only the MTT endpoint (as a readout for cell viability), but also the inflammatory mediator IL

1alpha was studied with regard to its predictive capacity as an adjunct to the MTT endpoint (Annexe 1, doc22). Upon completion of the ECVAM Skin Irritation Validation Study (Annexe 1 doc24, doc26), the EpiSkin test method using the MTT endpoint was found capable of reliably distinguishing irritant from non-irritant chemicals, while the Epiderm test method using the same exposure time as the EpiSkin (the so-called common protocol featuring 15 minutes exposure time to the test substance) was recommended for identification of positives only (ESAC 2007 in Annexe 2). The ESAC recommended further optimisation work of the EpiDerm assay which was conducted between spring 2007 and early 2008 and let to the update validation of the modified EpiDerm assay (see below). Therefore, the EpiDerm test method and associated protocol validated during the SIVS are hereinafter referred to as "original Epiderm test method". The EpiSkin's performance (as 'reference method') was used for specifying the ECVAM skin irritation Performance Standards with regard to the defined accuracy values (Annexe 1, doc09) in May 2007.

2) ***2008 - Validation of the modified EpiDerm and the SkinEthic assays:*** Two equivalence validation studies on the basis of the ECVAM skin irritation Performance Standards for *in vitro* skin irritation testing. (ESAC 2008, see Annexe 3). Both studies were conducted by industry and submitted to ECVAM for evaluation and peer review. The SkinEthic test method was regarded by ECVAM as sufficiently similar in comparison with the validated EpiSkin method and was hence evaluated as a similar method through a catch-up (me-too) validation study on the basis of the Performance Standards. In addition, ECVAM evaluated the test method in its in-house laboratory ('Correlate') with regard to transferability of the method (data see Annexe 5). The modification of this modified EpiDerm test method was restricted to the protocol / SOP: the exposure time was extended from 15 minutes to 60 minutes. The method was regarded sufficiently similar and admitted as an update validation study on the basis of the Performance Standards

3) ***2009 - Performance of all three methods under UN GHS (detailed information and data in section 0.5):*** In April 2009 the performance of three in vitro test methods validated by ECVAM was found satisfactory by the ESAC (ECVAM Scientific Advisory Committee) also for use in the context of classification decisions on the basis of the UN GHS system as applicable to all authorities, i.e. using one single irritant category ("category 2") (ESAC 2009, Annexe 4). All three methods (the EpiSkin, the EpiDerm and the SkinEthic) had previously been validated as capable of reliably distinguishing between no-label and R38 (irritant) substances under the previous EU classification system relating to the EU Dangerous Substance Directive (Directive 67/548/EEC). In December 2008, the EU adopted the UN GHS system through the adoption of the respective implementing Regulation on the Classification, Labelling and Packaging of Substances and Mixtures (CLP Regulation EC 1272/2008) which came into force on 20 January 2009 and will, after a transitional period, replace the previous EU legislation (the EU DSD system) for the classification of substances and mixtures (i.e. preparations).

0.3.1. RhE Skin Irritation Test Method Applicability

Recommendation 1 of the 2nd OECD Expert Consultation Meeting (ECM)(Washington, 15-17 June 2009) on the applicability of the RhE test methods:

"The RhE in vitro methods are empirical testing methods and directly address the initial step of the inflammatory cascade/mechanism of action (cell damage and tissue damage resulting in localized trauma). Therefore, there is no scientific reason to assume that these methodologies are not applicable to all

substances and mixtures, unless there is specific information that provides evidence regarding such limitations."

The WNT22 (Paris, 23-25 March, 2010) did not fully endorse the 1st recommendation of the OECD Expert Consultation Meeting on RhE Skin Irritation test method held in Washington, USA, on 15-17 June 2009, with respect to the conclusion on the potential applicability of the test method as stated in the second sentence of the recommendation:

"Therefore, there is no scientific reason to assume that these methodologies are not applicable to all substances and mixtures, unless there is specific information that provides evidence regarding such limitations"

The WNT is of the opinion that given the fact that even though the RhE skin irritation test method is a model system that, to a large extent, simulates the *in vivo* situation, it is possible that there may be differences between the *in vitro* test method and the in vivo situation. The WNT therefore recommends that test users should apply caution before using the RhE skin irritation test method for chemical classes that have not been tested in the RhE models since the accuracy of the *in vitro* test method has not been determined yet for such chemical classes. This concern includes the following chemical classes that have been identified to potentially contain irritants and which have not been included in the validation studies or optimisation phases of the RhE models in the RhE skin irritation test method:

- Enzymes
- Alkalis, including aliphatic amines
- Organic solvents, aromatic
- Oxidants (e.g. organic peroxides)
- Quarternary ammonium compounds
- Metal compounds

0.4 Acceptance Process by Regulatory Authorities: EU, OECD, the UN GHS system

In the EU the regulatory acceptance process of the RhE skin irritation methodology validated by ECVAM is already fairly advanced: A EU test method (B.46) on the basis of the three ECVAM-validated human reconstructed epidermis (RhE) models has received a positive statement from the EU regulatory committee for REACH (the EU's chemicals' regulation) and CLP. If the European Parliament agrees with this draft test method, B.46 will be included into the EU test method regulation (EC 440/2008). This is expected by late summer 2009. The EU test method regulation is the central piece of legislation regulating in EU Member States the use of canonized test methods for safety assessments of chemicals and cosmetics.

The OECD draft Test Guideline is currently under review for acceptance by OECD member state regulatory authorities for distinguishing no-category from category 2 (irritant) substances on the basis of the UN GHS classification using one single irritant category ("category 2"). This one category scheme is applicable to all authorities since it is (a) centrist in sensitivity among existing classifications, (b) recognizes the possible persistence of effects throughout the duration of the *in vivo* test and (c) acknowledges that animal responses may be quite variable (United Nations 2008; paragraph 3.2.2.5.1).

UN GHS allows the use of an additional 'opt-in' category for mild irritants ("category 3"). This additional category is however only applicable to some authorities (e.g. pesticides) that "*want to have more than one irritant category*" (United Nations 2008, paragraph 3.2.2.5.1 sub point c). In the EU, the UN GHS system as applicable to all authorities is directly transposed and implemented through the Regulation on the Classification, Labelling and Packaging of Substances and Mixtures (CLP Regulation EC 1272/2008) which was adopted in December 2008, came into force on 20 January 2009 and will, after a transitional period, replace the previous EU legislations for the classification of substances and mixtures (i.e. preparations). In agreement with the UN GHS system, the CLP system continues to use one irritant category.

However, according to the new rules for skin irritation classification and labelling (C&L), the cut-off score to distinguish between no-category and category 2 substances was shifted to 2.3 (UN GHS or CLP) from a value of 2.0 (previous EU system). Consequently substances with an *in vivo* score between 2.0 and 2.3 that were considered irritant under the previous EU system are now considered non-irritants under UN GHS, which does not use the optional Category 3. This shift and the various categories are schematically depicted in Figure 0.4.

The following abbreviations concerning the classification schemes are used in this text:

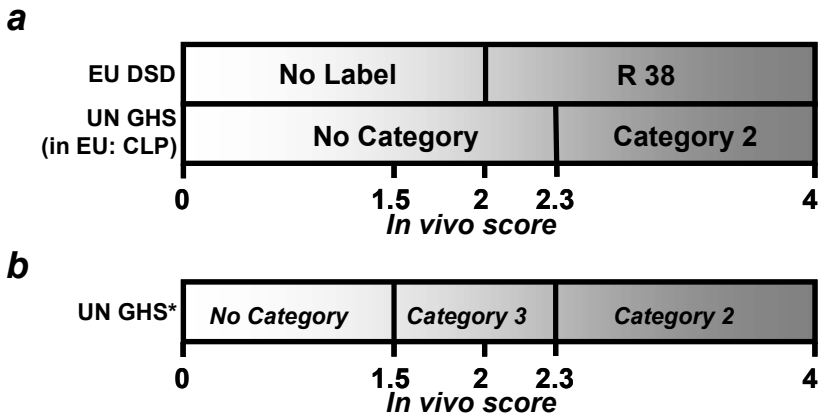
EU DSD The previous EU classification system based on the Dangerous Substance Directive (DSD) which is currently being phased out and replaced by the CLP system implementing UN GHS as applicable to all authorities. The EU DSD system used the risk phrase R38 to label irritant substances. Non-irritants were not labelled ('no label').

UN GHS The UN GHS system as applicable to all authorities (see table 3.2.2 in the UN GHS document), i.e. using only one irritant category ('category 2') to label substances found irritant by appropriate test methods. Non irritant substances are labelled as 'no category'. The UN GHS system as applicable to all authorities is directly transposed and implemented in the EU through the CLP regulation.

UN GHS* The UN GHS system as applicable to some authorities that wish to have more than one irritant category (e.g. pesticides, see UN GHS document section 3.2.2.5.4). The additional optional ("opt-in") category is called 'category 3'.

It is important to note that the current draft test guideline for *in vitro* skin irritation only addresses the irritancy part of the existing TG 404 including the irritation part of the test strategy outlined in the supplement of TG 404. It should be noted that the *in vitro* methodology now under discussion has been validated for corrosivity testing (using different protocols) and has been adopted as OECD TG 431 (*in vitro* skin corrosion using human skin models).

Figure 0.4: ***(a)*** *Comparison of the threshold values for skin irritation classification and labelling in the EU prior to adoption of the UN GHS system (abbreviated as EU DSD = European Classification System based on the Dangerous Substance Directive) and after the adoption of the UN GHS system as applicable to all authorities (UN GHS). In the EU the UN GHS system is implemented through the so-called CLP regulation (CLP). While the cut-off score of the ECS was 2.0 the cut-off score of the UN GHS (CLP) system is 2.3. Therefore, in the EU, all substances with scores from 0 to 2.3, previously considered irritant, will be 'no category' under UN GHS (CLP) if tested in the in vivo test system.* ***(b)*** *The UN GHS system as applicable to some authorities (UN GHS*) featuring an optional irritant category. According to the provisions of the GHS health hazard document, this optional Category 3 is available for "those authorities that want to have more than one skin irritant category" (United Nations 2008). This optional additional category is not implemented in the EU. A score of 0 means that the substance did not produce visible signs of irritation on rabbit skin. A score of 4 means that the substance produced the strongest possible visible response for one or two of the endpoints evaluated. This transition from non- irritant to irritant is indicated by the gradient from white to grey.*



0.5 Performance of the three validated methods under UN GHS

The performance of the validated test methods was examined in 2008 by ECVAM in the course of the EU acceptance process of EU test method B.46 (see section 0.5; Griesinger et al. 2008), an ESAC working group also charged with the adaptation of the original performance standards and operating from February to May 2009 and the ESAC in March 2009 (ESAC 2009 Annexe 4). The performance of all three tests under GHS-EU was calculated taking this shift of the cut-off value between no label/ no category and R38/category 2 into consideration. The cut off was moved by 0.3 score points from an *in vivo* score of 2.0 to 2.3 to provide a single irritant category "centrist in sensitivity among existing classifications" (United Nations 2008; paragraph 3.2.2.5.1 sub point a).

0.5.1 General considerations

Before considering the performance of the methods, it appears useful to revisit some basic concepts and definitions schematically depicted in Figure 0.5.1. Due to convention and agreement within the regulatory community, substances with specific in vivo score values are considered non-irritant (Actual Negatives, AN) or irritants (Actual Positives, AP). This convention gives rise to the classification and labelling rules within a specific context. The convention is based on the rabbit *in vivo* test which is assumed to produce test outcomes representing the "true" situation. During validation, reference *in vivo* data are thus regarded as the truth, against which the test result is measured and conclusions on the test result in relation to the "truth" (AP, AN) are drawn.

- Positive test results for substances regarded as Actual Positives (AP) are thus considered ***True Positives (TP)***, while Negative test results associated with AP are considered ***False Negatives (FN)***.
- Positive test results for substances regarded as Actual Negatives (AN) are consequently considered ***False Positives (FP)*** while Negative test results associated with AN are considered ***True Negatives (TN)***.

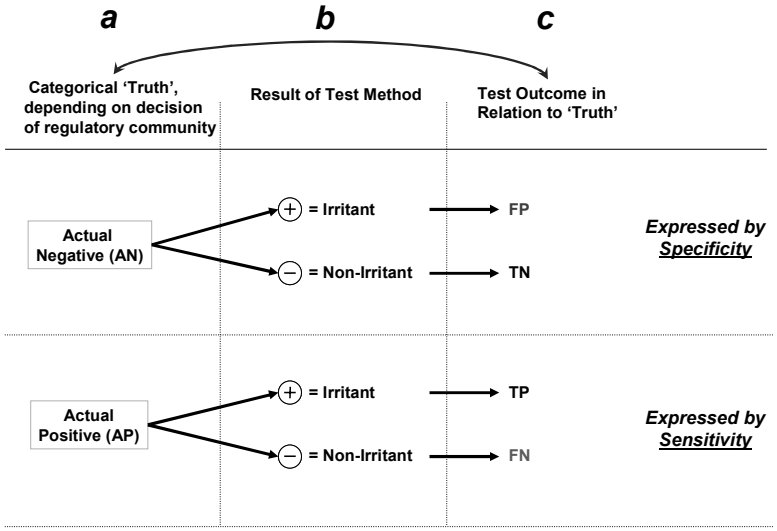
Consequently, any change in what is considered an Actual Negative (AN) and an Actual Positive (AP) will directly impact on what is considered a FP, TP, FN, TN.

In the current context this is the case due to the shift in the cut-off score: former APs (2. to 2.3) are now regarded AN. Consequently formerly correct positives (TP) are no considered FP, while formerly false negatives (FN) are now considered TN. This is depicted in Figure 0.5.2.

It is important to keep in mind that this attribution of whether a substance is regarded as AP or AN is to a great extent an arbitrary decision made by convention. Irritancy represents is a continuous band of reactions from non-irritant (no inflammatory reaction) to strongly irritant (strong inflammatory reaction). The categorisation represents the scientific (regulatory) community's way of looking at the data in a simplified way for good practical reasons.

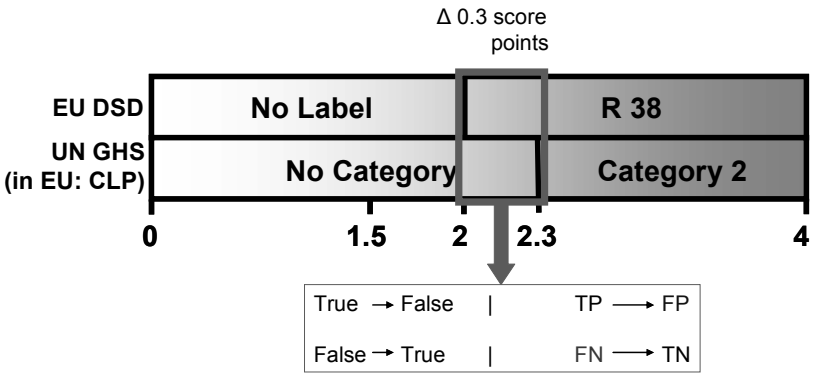
Consequently the changes observed at the 2.0 to 2.3 in vivo score band may be regarded as artifactual inasmuch as they do not change the biological test result which samples the true reaction of the tissue construct (or the skin) to the xenobiotic which is translated into a categorical decision through a Prediction Model (PM).

Figure 0.5.1 (a) *the categorical truth associated with a substance (either Actual Positive [AP] or Actual Negative [AN]) forms the basis – via the test result* ***(b)*** *– for the test outcome* ***(c)****: the test result is – depending on the convention in (a) considered a False Positive (FP), a True Negative (TN), a True Positive (TP) or a False Negative (FN).*



When considering and re-calculating the performance of the methods on the basis of (1) the SIVS for the EpiSkin method and (2) the equivalence studies using 20 PS RC for the modified EpiDerm and SkinEthic methods, it is important to realise that the predictions of the methods calculated under EU DSD for substances with scores from 0 to 2.0 and 2.3 to 4.0 remain correct since the categorical decision has not changed for these scores. These continue to be regarded as "non-irritant" (0-2.0) and "irritant" (2.3 – 4.0) and consequently the former test outcomes (TN, FP for ANs TP, FN for APs) remain correct. Only in the narrow band from 2.0 to 2.3 (delta 0.3 score points), the categorical decisions attached to the scores are changed. This is schematically summarised in Figure 0.5.2. In this band, formerly "true" test results become "false" and formerly "false" ones become "true", i.e. a **former True Positive (TP)** is now regarded as a **False Positive (FP)** and a **former False Negative (FN)** is now regarded as a **True Negative (TN)**.

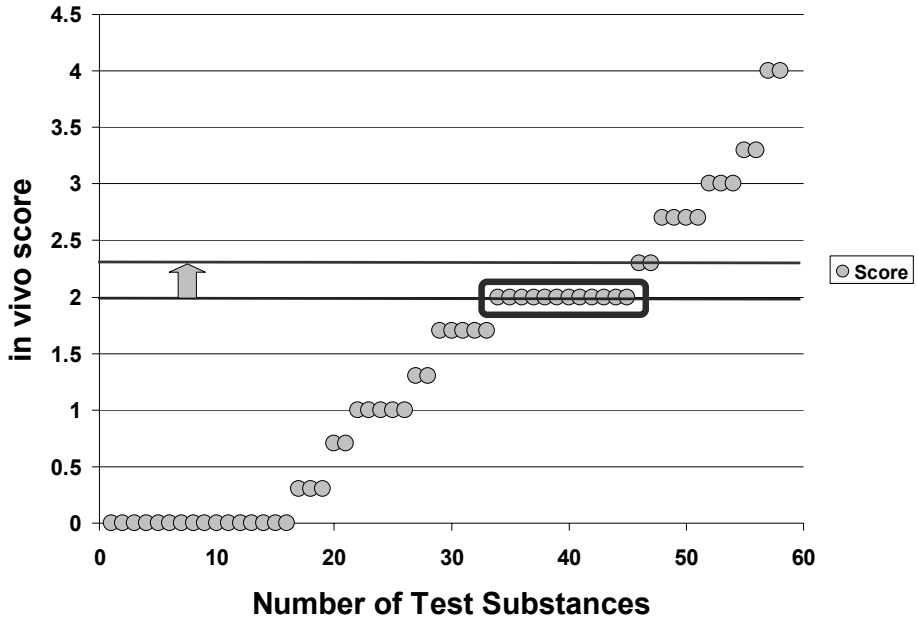
Figure 0.5.2 *Schematic depiction of the change in categorical test results in the narrow band from 2.0 to 2.3 in vivo scores due to the shift of the cut-off value from 2.0 (EU DSD) to 2.3 under UN GHS. Former TP are now considered FP, while former FN are now considered TN.*



0.5.2 Impact of the test chemical selection on the changed accuracy values under UN GHS

It is furthermore noteworthy that within the validation test substance set of the SIVS substances with scores within this 2.0 to 2.3 band are overrepresented. This is illustrated in Figure 0.5.3, a scatter plot of the in vivo irritancy scores of the validation substance set sorted from low to high irritancy scores.

Figure 0.5.3: Scatter plot of the in vivo scores of the test substances used in the SIVS. *The substance scores are distributed from 0 to 4.0. Evidently substances with a score of 0 and those with a score of 2.0 are overrepresented. The overrepresentation of score 0 substances is in agreement with the high prevalence of such substances in representative populations (prevalence may be as low as ca. 7.5% [EU DSD] or about 3% [UN GHS]). Score 2.0 substances however were overrepresented (red box) to allow assessment of the predictive accuracy of the test methods at the borderline zone. The cut-offs for EU DSD (purple) and UN GHS (red) are indicated.*



Out of the 26 irritant chemicals of the SIVS (scores ≥ 2.0), 12 substances had an in vivo score of 2.0 (cut-off score between no label and R38; red box in Figure 0.5.3), while 13 covered the further range from 2.3 to 4.0 (Figure 0.5.3). The latter group of substances was deemed sufficient to assess the accuracy and reliability of the methods for the irritant range, while a high number of borderline substances (score 2.0) was deliberately selected to challenge the test methods with regard to their accuracy of yielding correct predictions at the cut-off.

When considering this 2.0 to 2.3 band (all 12 substances had scores of 2.0!) where the attribution of AN and AP have changed due to new definition of the cut-off value, it becomes obvious that under the EU DSD system 5 out of 12 substances were FN (results on the basis of the mode of laboratory predictions and including all runs). Under UN GHS however, all of these 5 FN predictions become correct (TN). However the previous 7 correct predictions (TP) are now considered FP. This is shown in Table 0.5.1.

Thus

- 5 FN out of 12 substances under EU DSD turn into 5 TN under UN GHS while
- 7 TP out of the 12 substances under EU DSD turn into 7 FP under UN GHS.

This has obvious consequences on the accuracy (specificity and sensitivity and overall accuracy) of the test method. Since there are now 7 more FP amongst the AN population under UN GHS, the specificity decreases quite remarkably. In contrast, since 5 former FN are not any longer under the AP population, the sensitivity is markedly increased.

Table 0.5.1: *Predictions of the EpiSkin test method (SIVS) under EU DSD and UN GHS (all runs have been included). Columns:* ***1:*** *Nr of chemicals,* ***2:*** *in vivo score,* ***3:*** *EU DSD category,* ***4:*** *UN GHS category,* ***5:*** *UN GHS* category,* ***6, 8:*** *mode of laboratory predictions (1 is irritant;0 is non-irritant),* ***7:*** *Test outcome in relation to the AN and AP attribution of chemicals (arbitrary categorical decision) under EU DSD and* ***9:*** *under UN GHS.*

1	2	3	4	5	6	7	8	9	10
Nr.	Substances Identities	*In vivo* Score	EU DSD	UN GHS*	UN GHS	EU DSD Median	EU DSD FP - FN	UN GHS Median	UN GHS FP - FN
1	1-bromo-4-chlorobutane	0	no label	no category	no category	1	FP	1	FP
2	3-diethylaminopropionitrile	0	no label	no category	no category	0	TN	0	TN
3	3-mercaptohexanol	0	no label	no category	no category	1	FP	1	FP
4	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	0	no label	no category	no category	0	TN	0	TN
5	diethyl phthalate	0	no label	no category	no category	0	TN	0	TN
6	di-propylene glycol	0	no label	no category	no category	0	TN	0	TN
7	dipropylene glycol monobutyl ether	0	no label	no category	no category	0	TN	0	TN
8	3,4-dimethyl-1H-pyrazole	0	no label	no category	no category	1	FP	1	FP
9	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine-1-carboxylate	0	no label	no category	no category	0	TN	0	TN
10	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	0	no label	no category	no category	0	TN	0	TN
11	cyclohexadecanone	0	no label	no category	no category	0	TN	0	TN
12	naphthalene acetic acid	0	no label	no category	no category	0	TN	0	TN
13	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	0	no label	no category	no category	0	TN	0	TN
14	2-(formylamino)-3-thiophenecarboxylic acid	0	no label	no category	no category	0	TN	0	TN
15	silane A-1430	0	no label	no category	no category	0	TN	0	TN
16	triethylene glycol	0	no label	no category	no category	0	TN	0	TN
17	2,6-dimethyl-4-nitrobenzeneamine	0.3	no label	no category	no category	0	TN	0	TN
18	allyl phenoxyacetate	0.3	no label	no category	no category	0	TN	0	TN
19	isopropanol	0.3	no label	no category	no category	0	TN	0	TN
20	2-ethylhexyl 4-aminobenzoate	0.7	no label	no category	no category	0	TN	0	TN
21	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0.7	no label	no category	no category	0	TN	0	TN
22	3-chloro-4-fluoronitrobenzene	1	no label	no category	no category	1	FP	1	FP
23	4-methylthio-benzaldehyde	1	no label	no category	no category	1	FP	1	FP
24	capryl-isostearate	1	no label	no category	no category	0	TN	0	TN
25	methyl stearate	1	no label	no category	no category	0	TN	0	TN
26	phenylethylalcohol	1	no label	no category	no category	0	TN	0	TN
27	Mixture of isomers: diethyl cis-1,4-cyclohexanedicarboxylate; diethyl trans-1,4-cyclohexanedicarboxylate	1.3	no label	no category	no category	0	TN	0	TN
28	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)	1.3	no label	no category	no category	0	TN	0	TN
29	allyl heptanoate	1.7	no label	opt. cat. 3	no category	0	TN	0	TN
30	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	1.7	no label	opt. cat. 3	no category	1	FP	1	FP
31	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; 5-endo-decylbicyclo[2.2.1]hept-2-ene	1.7	no label	opt. cat. 3	no category	0	TN	0	TN
32	heptyl butyrate	1.7	no label	opt. cat. 3	no category	0	TN	0	TN
33	2-phenylhexanenitrile	1.7	no label	opt. cat. 3	no category	0	TN	0	TN
34	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	2	R38	opt. cat. 3	no category	1	TP	1	FP
35	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol; 56% (1'R,2R) & 40%(1'R,2S) isomer	2	R38	opt. cat. 3	no category	1	TP	1	FP
36	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	2	R38	opt. cat. 3	no category	1	TP	1	FP
37	hexyl salicylate	2	R38	opt. cat. 3	no category	0	FN	0	TN
38	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	2	R38	opt. cat. 3	no category	1	TP	1	FP
39	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]decan-2-ol	2	R38	opt. cat. 3	no category	1	TP	1	FP
40	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	2	R38	opt. cat. 3	no category	1	TP	1	FP
41	isostearic acid monoisopropanolamide	2	R38	opt. cat. 3	no category	0	FN	0	TN
42	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)	2	R38	opt. cat. 3	no category	0	FN	0	TN
43	terpinyl acetate	2	R38	opt. cat. 3	no category	0	FN	0	TN
44	tri-isobutyl phosphate	2	R38	opt. cat. 3	no category	1	TP	1	FP
45	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	2	R38	opt. cat. 3	no category	0	FN	0	TN
46	1-decanol	2.3	R38	category 2	category 2	1	TP	1	TP
47	cyclamen aldehyde	2.3	R38	category 2	category 2	1	TP	1	TP
48	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	2.7	R38	category 2	category 2	1	TP	1	TP
49	1-bromohexane	2.7	R38	category 2	category 2	1	TP	1	TP
50	α-terpineol	2.7	R38	category 2	category 2	1	TP	1	TP
51	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	2.7	R38	category 2	category 2	1	TP	1	TP
52	butyl methacrylate	3	R38	category 2	category 2	1	TP	1	TP
53	di-n-propyl disulphide	3	R38	category 2	category 2	0	FN	0	FN
54	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	3	R38	category 2	category 2	1	TP	1	TP
55	1-methyl-3-phenyl-1-piperazine	3.3	R38	category 2	category 2	1	TP	1	TP
56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	R38	category 2	category 2	1	TP	1	TP
57	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	4	R38	category 2	category 2	0	FN	0	FN
58	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	4	R38	category 2	category 2	1	TP	1	TP

0.5.3 Performance values under UN GHS

The performance of all three methods also under GHS-EU was found satisfactory (Table 1) by ESAC (ESAC 2009, see Annexe 4). While the specificity of the EpiSkin method is decreased from 81.8%* (EU DSD) to 71.1 %* (UN GHS), the test sensitivity has increased from 72%* (EU DSD) to 84.6%* (UN GHS). The two other methods show similar values for the specificity (both tests 69.2%*), and higher sensitivity values than the reference method under GHS-EU.

Table 0.5 *Accuracy values for the three ECVAM-validated skin irritation in vitro test methods under UN GHS as applicable to all authorities.*

Test performance parameter	EpiSkin test method (58 chemicals[1])	EpiSkin test method (20 reference chemicals[3])	Modified EpiDerm test method (20 reference chemicals[3])	SkinEthic test method (20 reference chemicals[3])
Specificity (%) [2]	**71.1**	**76.9**	**69.2**	**69.2**
Sensitivity (%) [2]	**84.6**	**85.7**	**85.7**	**100**
Overall Accuracy (%) [2]	**74.1**	**80**	**75**	**80**

[1]) The test substances from the ECVAM Skin Irritation Validation Study (SIVS) conducted from 2003 to 2007.
[2]) Based on the median (or mode) of the individual laboratory predictions.
[3]) Original 20 RC from the ECVAM Performance Standards May 2007

ESAC concluded that the original ESAC statements relating to these test methods therefore continue to be accurate with respect to the scientific validity of the methods also under the GHS-EU classification system. ESAC concluded that the use of the test methods for purposes of classification and labelling can now be extended to the UN GHS system as applicable to all authorities (i.e. using one irritant category) (ESAC 2009 Annexe 4).

When considering the changes in performance (accuracy) of the three validated test methods, two aspects should be taken into account:

*1) Due to the relative **overrepresentation** of score 2.0 substances (12 out of 58), the impact of the cut-off shift on the specificity and sensitivity values is **greater than expected** considering that the change concerns only Δ 0.3 score points.*

*2) Since the **test methods were optimised for a cut-off score of ≥2.0**, predictions for chemicals of this non-irritant score group tend to tilt towards positive predictions (although under EU DSD 5 substances out of 12 at this score were FN). This propensity to make positive predictions already at score 2.0 (7 substances out of 12 at 2.0 reported as FP under UN GHS) means however that, under UN GHS the test methods recognise AP with high sensitivity from score 2.3 onwards. This may support an application of the test methods in agreement with the **precautionary principle**.*

The performance of the validated methods under UN GHS was originally calculated in reference to the *in vivo* scores calculated under EU DSD provisions. In response to a comment from NL, the *in vivo* scores were carefully recalculated on the basis of the provisions of the UN GH system in order to assess whether the reference scores associated with the test substances would change which would lead to possible consequences with regard to the classification decisions and, ultimately, influence the recalculated predictive values of the *in vitro* methods. However, recalculation showed (see section 4 for details) that the scores associated with the individual testing chemicals were unchanged.

Thus, the original *in vivo* scores of the test substances calculated under EU DSD and the classifications yielded on the basis of these continue to be correct also under UN GHS. Consequently the recalculated performance values for the in vitro methods are correct.

►Recommendation Nr. 2 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, 15-17 June 2009) on GHS classification and test method performances:

The OECD ECM is of the opinion that the in vivo classifications used for the recalculation with respect to the UN GHS classification system are correct and consequently the stated test method performances are adequate.

0.6 Update of the performance standards (PS) for in vitro skin irritation testing

Due to the new definition of the cut-off value for distinguishing no category from category 2 (irritant) substances, an update of the original ECVAM PS (Annexe 1 doc09) became necessary since these were based on the categorical decision rules under EU DSD. The update concerned first and foremost (1) the target accuracy values that should be met by new similar (me-too) or modified test methods and (2) the 20 Reference Chemicals (RC). The PS of the current OECD draft TG on *in vitro* skin irritation are based on the updated ECVAM PS. It is acknowledged that this update owes much to the work of the 1st and 2nd OECD ECM in Berlin (2008) and Washington (2009).

0.6.1 Reference Chemicals (RC)

The RC needed to be updated for two reasons:

1) Under UN GHS the performance of the methods with regard to the accuracy of yielding correct (TP, TN) predictions had changed. These changes required an updated of the 20 Reference Chemicals (RC) in order to reflect the performance of the validated reference methods (EpiSkin) under UN GHS on the basis of 20 RC when taking all factors that led to the new predictivity figures as calculated under UN GHS (e.g. overrepresentation of score 2.0 substances) into account.
2) The 20 RC were not balanced any longer with respect to an equal distribution of irritants and non irritants (Table 0.6.1).

In summary RC were deleted and substituted by alternative RC to:

a) map the performance of the VRM under UN GHS in the RC set
b) maintain a balanced set of RC and moreover
c) take into account the commercial availability of RCs in member countries and
d) delete substances (as far as possible) that are irritant in rabbit but non-irritant in human

The rationale for deleting and inserting substances is briefly outlined in Table 0.6.2. All changes of the RC set are described in more detail in section 4 of this background document.

In total 6 of the previous chemicals were deleted (yellow in Table 0.6.1) while 6 new chemicals were inserted (orange in Table 0.6.1), which resulted in the reduction of the number of UN GHS Cat 2 substances (based on the rabbit test) that are known to be non-irritant in humans (on the basis of human patch-test data). These substances are labelled with an asterisk in Table 0.6.1. The new test substances moreover reflect the new accuracy values of the reference method on the basis of the 58 substances, in particular when considering new rules for calculating the method performance as specified in the updated ECVAM Performance Standards and those of the current draft Test Guideline (see section 0.6.3 and section 4).

Due note should be taken of the fact that these substitutions were difficult to achieve due to the low prevalence of irritant substances (e.g. irritants within EU NCD about 5% under EU DSD and about 3% under UN GHS). Moreover, RCs do have to fulfil the criteria set out in section 2 (e.g. high quality *in vivo* reference data) based on the provisions of the OECD guidance document Nr. 34 on validation. This led to a further reduction of eligible candidate RCs.

The shortage of appropriate and suitable substances ultimately led to the situation that (a) three of the new RC have not been part of the original testing set of the ECVAM SIVS and that (b) four of the twenty

RC have been tested in the validated reference method (EpiSkin) in only one laboratory but not in three laboratories as the remainder of the RC, directly derived from the SIVS testing set (heptanal was not part of the SIVS testing set but was already included in the original list of RC).

However, it is important to note that

a) all four RCs have been tested in **three validated test methods** in at least three runs (consisting of three tissue replicates) in one to maximally three laboratories, and;

b) all **predictions yielded were 100% concordant** between tissue replicates, runs and the three test methods (see Table 4.3 in section 4) and **correct** (true positive; TP), and;

c) **none of the RC has been used for either development or optimisation** of any of the three test methods (i.e. they have not been part of the training set).

Finally, it should be noted that the new RC are in full agreement with the provisions and recommendations of the OECD Guidance Document Nr. 34 on the validation of testing methods which allows (1) the use of substances for the evaluation of the validity of test methods that were not part of the original testing set and (2) stipulates that predictive capacity may be evaluated in less than the recommended three laboratories. The relevant passages are reproduced below:

> **Paragraph 41:** *"If any of the recommended chemicals are unavailable,* ***other chemicals for which adequate reference data are available could be substituted.*** *[…] However, these additional chemicals should not include any that had been used to develop the proposed test method."*
>
> **Paragraph 82:** "*However, the assessment of the predictive capacity (performance of the test method) may be conducted in fewer laboratories if the data are adequate."*

In summary all proposed Reference Chemicals in this draft OECD TG have been tested in all three test methods. Some (n=4) of the newly proposed Reference Chemicals were tested in a single laboratory for some test methods but all of these were 100% concordant between replicates, runs and the three test methods (see section 4). Moreover, all three test methods showed acceptable reproducibility both within (> 90%) and between (> 85%) laboratories during their validation, which means that the data from a single laboratory should be sufficient to evaluate their performance regarding these Reference Chemicals.

Recommendation Nr. 3 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, 15-17 June 2009) on the Reference Chemicals (RC) selection:

Acknowledging the low prevalence of suitable irritant chemicals and other applicable restrictions that result in a low number of eligible candidate RC, the OECD ECM recommends and endorses the proposed new RC which take up the changes necessitated by the UN GHS system since sufficient supporting testing information is available to substantiate the appropriateness of this RC set for purposes of Performance Standards-based validations and since the proposed RC set is in agreement with the provisions of the OECD Guidance Document Nr. 34 on the validation of testing methods.

Table 0.6.1 Original and updated reference chemicals. *At the bottom of the table are the accuracy values of the EpiSkin reference method for these substances. Yellow=deleted substances. Orange=inserted substances. Bold and asterisk=substances known to be non-irritant in humans. The EpiSkin classification decision is crucial since EpiSkin is the validated reference method (VRM) with respect to the accuracy target values stipulated in the Performance Standards (PS).*

Nr.	Original Reference Chemical	*In vivo* Score	*EU DSD*	*UN GHS*	EPISKIN classifi-cation	Updated Reference Chemical	*In vivo* Score	*EU DSD*	*UN GHS*	*UN GHS*
1	1-bromo-4-chlorobutane	0	no	no cat.	**FP**	1-bromo-4-chlorobutane	0	no	no cat.	**FP**
2	diethyl phthalate	0	no	no cat.	TN	diethyl phthalate	0	no	no cat.	NI
3	di-propylene glycol	0	no	no cat.	TN	naphthalene acetic acid	0	no	no cat.	NI
4	naphthalene acetic acid	0	no	no cat.	TN	allyl phenoxy-acetate	0,3	no	no cat.	NI
5	allyl phenoxy-acetate	0,3	no	no cat.	TN	isopropanol	0,3	no	no cat.	NI
6	isopropanol	0,3	no	no cat.	TN	4-methyl-thio-benzaldehyde	1	no	no cat.	**FP**
7	4-methyl-thio-benzaldehyde	1	no	no cat.	**FP**	methyl stearate	1	no	no cat.	NI
8	methyl stearate	1	no	no cat.	TN	heptyl butyrate	1,7	no	opt. cat. 3	NI
9	allyl heptanoate	1,7	no	opt. cat. 3	TN	hexyl salicylate	2	R38	opt. cat. 3	**NI**
10	heptyl butyrate	1,7	no	opt. cat. 3	TN	cinnamaldehyde	2	R38	opt. cat. 3	**FP**
11	hexyl salicylate	2	R38	opt. cat. 3	**FN**	**1-decanol ***	2,3	R38	cat. 2	TP
12	terpinyl acetate	2	R38	opt. cat. 3	**FN**	cyclamen aldehyde	2,3	R38	cat. 2	TP
13	tri-isobutyl phosphate	2	R38	opt. cat. 3	TP	1-bromohexane	2,7	R38	cat. 2	TP
14	**1-decanol***	2,3	R38	cat. 2	TP	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	2,7	R38	cat. 2	TP
15	cyclamen aldehyde	2,3	R38	cat. 2	TP	5% potassium hydroxide	3	R38	cat. 2	TP
16	1-bromohexane	2,7	R38	cat. 2	TP	**di-n-propyl disulphide***	3	R38	cat. 2	**FN**
17	**a-terpineol***	2,7	R38	cat. 2	TP	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3,3	R38	cat. 2	TP
18	**di-n-propyl disulphide***	3	R38	cat. 2	**FN**	1-methyl-3-phenyl-1-piperazine	3,3	R38	cat. 2	TP
19	**butyl methacrylate***	3	R38	cat. 2	TP	heptanal	3,4	R38	cat. 2	TP
20	heptanal	3,4	R38	cat. 2	TP	Tetrachloroethylene	4	R38	cat. 2	TP
Specificity					**80**					**70**
Sensitivity					**70**					**90**
Accuracy					**75**					**80**

Table 0.6.2 *Summary of changes of the Performance Standard Reference Chemical Set as discussed and agreed during the OECD ECM meeting Nr. 1 in Berlin, Germany (22-23 October 2008) and the ECM meeting Nr. 2 in Washington D.C. (15-17 June 2009).*

Original substance (Nr. of original RC; *in vivo* score)	**Reason for deletion**	**New substance (Nr. of original RC; *in vivo* score)**	**Reason for insertion**
tri-isobutyl phosphate (Nr. 13, score 2.0)	Substance not available in Japan	**Cinnamaldehyde** (Nr. 10; score 2.0)	This substance is available in Japan, has the same score as the deleted substance and is also a FP with EpiSkin. Only available option.
di-propylene glycol (Nr. 3; score 0)	Deletion of superfluous NI to achieve a balanced set of irritants / non-irritants in the RC under UN GHS	**2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl** (Nr. 14; score 2.7)	Insertion of missing irritants to arrive at balanced set of irritants/non- irritants in the RC under UN GHS. Selected from the SIVS list.
allyl heptanoate (Nr. 9; score 1.7)	Deletion of superfluous NI to achieve a balanced set of irritants / non-irritants in the RC under UN GHS	**Benzenethiol, 5-(1,1-dimethylethyl)-2-methyl** (Nr. 17; score 3.3)	Insertion of missing irritants to arrive at balanced set of irritants/non- irritants in the RC under UN GHS. Selected from the SIVS list.
terpinyl acetate (Nr. 12; score 2.0)	Deletion of superfluous NI to achieve a balanced set of irritants / non-irritants in the RC under UN GHS	**1-methyl-3-phenyl-1-piperazine** (Nr. 18; score 3.3)	Insertion of missing irritants to arrive at balanced set of irritants/non- irritants in the RC under UN GHS. Selected from the SIVS list.
alpha terpineol (Nr. 17; score 2.7)	Non irritant in humans	**Potassium Hydroxide (5% aq.)** (Nr. 15; score 3)	Only available option (see section 4).
Butyl methacrylate (Nr. 19; score 3.0)	Non irritant in humans	**Tetrachloroethylene** (Nr. 20; score 4.0)	Only available option, since the possible alternative 1,1,1-trichloroethane is listed in the Montreal Protocol (see section 4)

0.6.2 Update of the accuracy target values

On the basis of the performance of the reference method with the 58 substances, the 20 RC and considerations of relevance in the species of interest the final predictive target values that should be met by new similar/me-too and modified methods are:

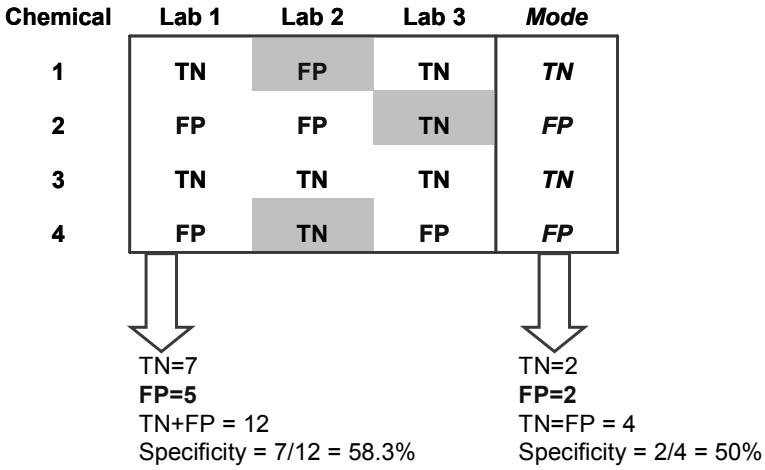
	Defined Accuracy Values
Specificity (%)	70
Sensitivity (%)	80
Overall Accuracy (%)	75

It is important however that a specific restriction applies to the 80% sensitivity target value: only two substances may be predicted as FN by more than one participating laboratory among the irritant RC group: *1-decanol* and *di-n-propyl dishulphide* (when considering the mode or median of laboratory predictions). Both substances are non-irritants in humans and it would be unscientific to punish test methods based on *human* keratinocytes for predicting these substances correctly in the species of interest.

0.6.3 New procedure for calculating the predictive capacity based on individual laboratory predictions

One other important change of the PS is briefly touched upon here: the way of calculating the accuracy values of the test method. Previously the accuracy (sensitivity / specificity) were calculated on the basis of the mode/median of individual laboratory predictions. However, considering the real application of the test after validation, testing will normally be performed only in on single laboratory. Hence it is more appropriate to calculate the performance on the basis of the individual laboratory predictions. This is illustrated in Figure 0.6.3.

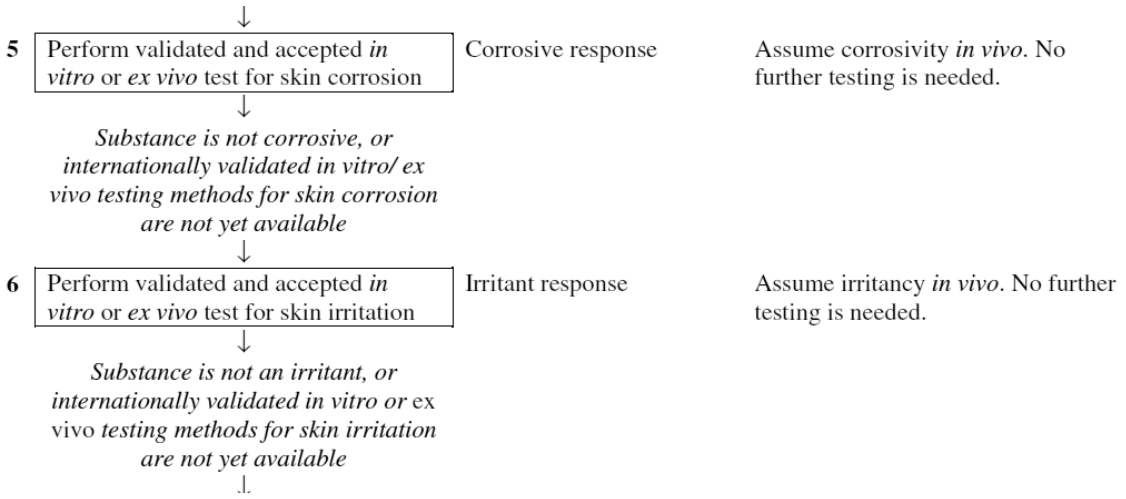
Figure 0.6.3: *Schematic depiction of two ways of calculating the predictive capacity (specificity, sensitivity, overall accuracy) of a test method, with specificity chosen as example here. The specificity can be calculated on the basis of the mode/median of predictions obtained in each laboratory for each chemical. In this example, the specificity is 50%. Note that the grey predictions are not taken into account in this calculation since they are overruled when calculating the mode (or median). In contrast, the specificity can be calculated on the basis of the individual laboratory predictions. In this case every prediction made is duly taken into account. The specificity in this example is 58%.*



0.7 Possible amendments of existing OECD TGs addressing skin corrosion/irritation as a consequence of a possible adoption of the draft OECD TG on *in vitro* skin irritation

As obvious from its title, the current OECD test guideline 404 for acute dermal irritation/corrosion addresses both, human health endpoints, skin corrosion as well as irritation. The supplement to TG 404 moreover outlines a testing strategy for detecting the skin corrosion and irritation potential of xenobiotics. This strategy suggests, among other methods, the use of *in vitro* skin corrosion and *in vitro* skin irritation methodology (Figure 0.7.1 copied from the 404 supplement).

Figure 0.7.1 *Part of the supplement to OECD TG 404 outlining the possible use of in vitro skin corrosion and skin irritation methods within a testing strategy to evaluate possible corrosivity/irritancy of xenobiotics*



Importantly, the current OECD draft test guideline on *in vitro* skin irritation addresses ***only*** the skin irritation part of OECD TG 404. Equally the existing OECD guidelines on alternative skin corrosion test methods (TG 430 on the basis of Transcutaneous Epithelial Resistance, TG 431 on the basis of reconstructed human epidermis and TG 435 on the basis of the Corrositex assay) address only the skin corrosivity part of the OECD TG 404. This is outlined in Table 0.7.2. Therefore, taken together, the TGs on alternative skin corrosion testing and the current draft TG on *in vitro* irritation do address the *empirical testing modules* of the OECD TG 404 testing strategy (but not other methodologies suggested in the test strategy, such as pH measurements or *in silico* approaches).

However, the relevant existing OECD TGs on skin corrosion are not mentioned or referenced in this strategy. Similarly, it is not clear from the existing TGs on corrosion (430, 431 and 435) that these address step 5 of the testing strategy outlined in the TG 404 supplement. Thus, at present, the possible interrelation between the TGs on (1) alternative skin corrosion, (2) the TG 404 and, pending adoption, (3) the draft OECD TG on skin irritation, is not outlined in any of these documents.

Clearly, to provide better guidance on strategic testing and the use of the appropriate methodologies, it would be propitious to provide mutual references in the relevant three sets of documents (404 *in vivo* corrosion/irritation AND 430,431,435 skin corrosion AND draft TG skin irritation).

Table 0.7.2 *Acute dermal human health endpoints addressed by the various OECD TGs and the draft TG under discussion within this context.*

Human health effect	**OECD 404**	**OECD TG 430, 431, 435 (*in vit*ro skin corrosion)**	**OECD draft TG (*in vitro* skin irritation)**
Skin corrosion	**Addressed**	**Addressed**	**Not addressed**
Skin irritation	**Addressed**	**Not addressed**	**Addressed**

To link the various existing documents in agreement with the OECD TG 404 supplement and to communicate clearly and unmistakable which of the human health effects are being addressed by which methodology the following updates may be adequate:

1) Inserting a reference to the skin corrosion TGs (430, 431 and 435) at step 5 (*in vitro* skin corrosion) of the OECD TG 404 supplement.
2) Pending adoption, inserting a reference to the draft skin irritation TG at step 6 (*in vitro* skin irritation) of the supplement to OECD TG 404.
3) Inserting an alert within TG 430, 431 and 435 that the TGs constitute test methods useful for addressing the skin corrosivity part (step 5) outlined within the OECD TG 404 supplement.
4) Inserting an alert within the present draft TG on *in vitro* skin irritation, that this TG constitutes a mean for addressing the *in vitro* skin irritation component of the 404 supplement (step 6).

Recommendation 4 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, June 15-17) on the revision of OECD Test Guidelines (TG) 404, 430, 431 and 435:

Pending the adoption of the draft OECD TG on in vitro skin irritation, the OECD ECM recommends a review and possible update of the OECD TG 404 on skin irritation/corrosion and TG 430, 431 and 435 on skin corrosion.

1. DETAILED BACKGROUND TO THE ECVAM VALIDATION STUDIES

1.1 Summary

Three *in vitro* skin irritation test methods based on Reconstructed human Epidermis (RhE) technology are currently under review at OECD level for acceptance by member state regulatory authorities. These test methods are able to reliably distinguish non-irritants from irritant substances on the basis of the UN GHS classification scheme using one single irritant category as implemented for all authorities (UN GHS guidance). In the EU, the GHS system is directly transposed through the Classification, Labelling and Packaging (CLP) regulation (EC 1272/2008).

The three assays are 1) the EpiSkin™, 2) the modified EpiDerm™ and 3) the SkinEthic RHE™ test method. The methods were validated in two groups of validation studies:

1) The ECVAM Skin Irritation Validation Study (SIVS), conducted from 2003 to 2007, an ECVAM-sponsored prospective validation study involving the blind testing of 58 test substances representing a wide spectrum of chemical functionalities and representing the full range of dermal irritancy (Annexe 1 doc 26; doc27).

2) Two equivalence validation studies on the basis of the ECVAM skin irritation Performance Standards for *in vitro* skin irritation testing.

The EpiSkin and EpiDerm assays have undergone formal ECVAM validation from 2003 – 2007 involving multi-laboratory testing of 58 substances of a wide spectrum of chemical functionalities and representing the full range of dermal irritancy (Annexe 1 doc26; doc27). In 2007 the EpiSkin was considered valid by ESAC as a full replacement test and its performance as reference method (Ref. 2) with regard to the predictive values was used for specifying the ECVAM skin irritation Performance Standards in May 2007. Originally validated for use in a testing strategy for the identification of positives only (Ref. 2), the EpiDerm test methods protocol was subsequently modified.

In November 2008, the modified EpiDerm and the SkinEthic test methods were validated on the basis of the ECVAM Skin Irritation Performance Standards (Ref. 3). As the EpiSkin assay, also the modified EpiDerm and the SkinEthic assay were found reliable and relevant test methods capable of distinguishing non-irritants from irritants and may therefore fully replace the traditional skin irritation test. It should be noted that conclusions on the applicability domain of the three methods rest mainly on the optimisation and validation data set. All three methods are valid for the classification of substances for skin irritancy according to CLP criteria (Ref. 4)

1.2 Background to the ECVAM Skin Irritation Validation Study (SIVS)

1.2.1 Prevalidation and Optimisation Studies leading to the SIVS

In 1998, the ECVAM Skin Irritation Task Force published a report on the actual status of *in vitro* skin irritation testing and proposed 10 "challenge chemicals" for which promising, concordant *in vivo* data from the rabbit test, *in vivo* data from 4hr human patch test, and *in vitro* data from the human skin model EpiDerm were available. Proponents of new *in vitro* test systems were encouraged to submit data obtained with new *in vitro* skin irritation test protocols for these chemicals for assessment whether these tests could be considered in an ECVAM prevalidation study (Annexe 1 doc15). At the same time the suitability of various endpoints for prediction of human skin irritation was evaluated in an EU 4th framework collaborative project in several reconstructed human skin models, revealing cell viability reduction (MTT reduction) and IL-1α release the most promising endpoints. Because MTT reduction and IL-1α release showed a high inter-correlation, and IL-1α release was more variable, MTT-reduction was proposed to be the best endpoint for human skin models (Annexe 1 doc16).

Of the test systems for which data were submitted to the ECVAM TF, five tests [perfused pig-ear, Prediskin, SIFT, EPISKIN, EpiDerm] had been considered promising for participation in the ECVAM prevalidation study. However, during the prevalidation study, two tests failed already in phase 2 due to insufficient reproducibility, whereas the other tests [SIFT, EPISKIN and EpiDerm] showed a sufficient intra- and inter-laboratory reproducibility, but failed in their ability to correctly predict the skin irritation potential of 20 chemicals that were tested in phase 3 of the ECVAM prevalidation study (Annexe 1 doc17). The ECVAM Management Team of the study therefore proposed refinement and optimisation of these three tests before considering them for formal validation.

In 2001, the ECVAM Skin Irritation Task Force and the laboratories responsible for the refinement of the tests met again and discussed ways forward to approach formal validation. In addition, since a post hoc analysis of prevalidation data for MTT reduction for EPISKIN and EpiDerm revealed similar sensitivity, it was recommended to develop a common test protocol for both skin models before the start of a formal validation study (Annexe 1 doc 18).

In November 2002, the ECVAM Skin Irritation Task Force (TF) discussed the refinements of the SIFT and the skin model tests and came to the conclusion that processing the tests to formal validation could be recommended. However, because all refinements were made using the 20 chemicals from the prevalidation study, the TF recommended to perform the SIVS in two phases: a first phase (phase 1) for the confirmation of the refinements made by the three leading labs by testing new chemicals in a controlled way under blind conditions. If the outcome of phase 1 were still promising, the tests would proceed to a second phase (phase 2), i.e. in a blind trial involving three laboratories per test.

During 2003, the EPISKIN test was further refined by one of the lead laboratories by extending the post incubation period of the tissues (after 15 min chemical exposure) to 42 hours which allowed significant effects to increase, and recovery from weak effects.

In May 2003, an ECVAM Stakeholder Workshop involving also regulators recommended to conduct a formal validation study and to concentrate primarily on the predictions of the EU classification system (R38 vs. no label), because the tests were developed and optimised for this classification scheme. The lead laboratories for EPISKIN and EpiDerm collaborated then in developing a common test protocol to be used in the ECVAM SIVS, and evaluated it first with the 20 "challenge" chemicals of the ECVAM prevalidation study. In 2004, upon request of the ECVAM SIVS Management Team and in parallel to performing phase 1 of the SIVS, the database was further increased by testing all non-corrosive chemicals recommended in the ECETOC reference data base (ECETOC report No. 66). The data obtained in both skin models with the optimised common protocol were very promising, and published back to back in 2005

(Annexe 1 doc19, 20). The study started formally with the 1st Meeting of the SIVS Management Team (MT) on 17-18 November 2003.

1.2.2 The ECVAM Skin Irritation Validation Study (SIVS)

After extensive optimisation and prevalidation activities (see background to the SIVS here above), ECVAM launched a formal validation study on three in vitro test systems in 2003. Two of the assays employed reconstituted human epidermis models (EPISKIN, EpiDerm) and one, the skin integrity function test (SIFT) employed ex vivo mouse skin. The aim of the study was to replace the regulatory Draize skin irritation test (EU B. 4 method; OECD TG 404) currently performed on albino rabbits by assessing the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of these test systems with a set of 58 coded test chemicals. The goal of the study was to evaluate if the *in vitro* tests would predict *in vivo* classification according to the EU classification system using the risk phrase R38 for skin irritants and no classification for non irritants. In addition, the chemical selection was representative for the three categories [strong (category 2), mild (category 3) and non-irritants (no category)] of the Globally Harmonised classification System (GHS) for permitting a post-hoc evaluation of the results according to GHS. The validation study was conducted according to the principles and criteria documented in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34). Furthermore, to ensure a high quality of the commercially produced human skin models, the facilities of the producers of the human skin models EPISKIN and EpiDerm were evaluated by independent auditors at the beginning of the ECVAM Skin Irritation Validation Study (SIVS).

A Chemicals Selection Sub-Committee (CSSC) was appointed to identify test chemicals to be used in the SIVS having high quality existing *in vivo* data with which to correlate the *in vitro* measurements. Since chemicals from the European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC) database of reference chemicals for skin irritation/skin corrosion had been extensively used in the preceding studies, the CSSC was requested to make use of novel sources for potential test chemicals. For this purpose chemicals were selected from the New Chemicals Database (NCD) which was the central archive within the EU notification scheme for new commercial chemicals. In addition, existing chemicals readily available from major manufacturing and/or distribution sources were selected from alternative databases such as the Toxic Substance Control Act (TSCA) database maintained by the US Environmental Protection Agency (EPA) and the ECETOC database, excluding those chemicals used in the previous optimisation and prevalidation phases.

A total set of 58 chemicals comprising a set of 25 existing chemicals and 33 chemicals from the NCD were selected and tested in the SIVS. The selected chemicals (a) represented statistically justified sample sizes for distinguishing R38 from non classified chemicals, (b) provided a balanced representation of the three GHS categories to allow for post-hoc evaluation of the performance of the assays for that classification system, and (c) accounted as far as possible the large prevalence known to exist for chemicals which have oedema and erythema scores of 0. These chemicals were independently coded and distributed to the participating laboratories. The selected chemicals presented a variety of molecular structures, functional chemical groups, effect and use categories, as well as a wide range of physical-chemical properties. They represented a challenging set of chemicals relevant to current industrial commerce for the alternative methods being validated.

In phase 1 of the ECVAM SIVS, 20 chemicals (9 irritant, 11 non irritant) from NCD were tested under blind conditions in the lead laboratories (EPISKIN - L'Oreal, EpiDerm - ZEBET, SIFT - Syngenta). The methods applied (with Standard Operating Procedures, SOP's) were the refined, optimised protocols developed after the ECVAM prevalidation study. For the human skin model assays this consisted in applying the test chemicals to the surface of the skin for 15 minutes, followed by a post-treatment incubation period of 42 hours, and the subsequent assessment of their effects on cell viability by using the MTT assay.

The prediction model related to MTT used in Phases 1 and 2 was the following:

"The test substance is considered to be irritating to skin (R38), if the tissue viability after exposure and post incubation is less or equal (≤) to 50%".

When cell viability (MTT reduction) was used as endpoint, the two skin models met the acceptance criteria set by the MT of the study. While the specificity (correct prediction of non-irritants) of both the EPISKIN and EpiDerm assays was 91%, their sensitivity (correct prediction of R38 irritants) was 67% and 56%, respectively. However, since almost all of the misclassified chemicals were lying at the threshold between irritant and non-irritant chemicals according to the EU classification scheme, the MT concluded that the predictive capacity of both Epiderm and EPISKIN was sufficient to justify them to proceed to Phase 2. On the other hand, the predictive capacity of the SIFT method was considered inadequate. For SIFT, it was suggested that the lead laboratory re-evaluated the test protocol and prediction model, particularly in relation to the manner in which solids and non-surfactant materials are handled.

In Phase 2, all 58 chemicals were assessed in three different laboratories for each of the two reconstituted human skin methods. Chemicals were re-coded from Phase 1 to ensure blind testing. The main endpoint measured for both Epiderm and EPISKIN was cell viability measured by MTT reduction, as used in all previous testing. However, a second endpoint, interleukin-1α release, was added for those chemicals which did not reduce cell viability below the threshold for predicting irritancy, to determine if it could be used to improve the sensitivity of the assays. This second endpoint was used in all three laboratories assessing EPISKIN and by the lead laboratory for Epiderm.

The prediction model related to the combined use of MTT and IL-1α release in Phase 2 was the following:

The test substance is considered to be **irritant** to skin: if the viability after 15 minutes of exposure and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1α release is more (>) than 60pg/ml.
The test substance is considered to be **non irritant** to skin: if the viability after 15 minutes of exposure and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1α release is less or equal (≤) to 60pg/ml.

The predictive capacities of the assays in this second phase are shown in Table 1 The within-laboratory reproducibility of classifications over three independent experiments meeting the acceptance criteria was 93.9% for EPISKIN (MTT) and 96.0% for EpiDerm (MTT). The between-laboratory reproducibility measured as the proportion of identical median classifications between laboratories was 89.5% for EPISKIN (MTT) and 88.5% for EpiDerm.

Table 1.1. *Predictive capacities of EPISKIN and EpiDerm (MTT: based on the median classification per laboratory; MTT+IL-1α: based on the classification derived from the mean viability of the independent experiments per chemical and laboratory)*

	EPISKIN (MTT)	***EPISKIN (MTT+IL-1α)***	***EpiDerm (MTT)****
Sensitivity	**74.7%**	**90.7%**	**57.3%**
Specificity	**80.8%**	**78.8%**	**83.8%**
Concordance/Accuracy	**78.2%**	**83.0%**	**72.4%**

*The addition of IL-1α to the EpiDerm protocol gave no improvement to the outcome

The study was forwarded to the ECVAM Scientific Advisory Committee (ESAC) with a proposal that EPISKIN could be considered as a replacement for the rabbit skin irritation method and Epiderm as a constituent of a testing strategy. In summary, further to an independent ESAC peer review, the ESAC concluded on the following (ESAC 2007 Annexe 2):

"The EPISKIN method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement ***(based on the performance of the assay as specified in the annex ESAC statement I)*** *for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances. At the present time, the IL-1α endpoint should be regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint.*

At this time, due to its high specificity, the EpiDerm model reliably identifies skin irritants, but negative results may require further testing (e.g. according to the tiered strategy, as described in the OECD TG 404). Improvement of the EpiDerm protocol should be made to increase the level of sensitivity".

1.3 Background to two validation studies based on the performance standards: modified EpiDerm and SkinEthic test method

1.3.1. Background to Validation Studies based on Performance Standards

The ECVAM Performance Standards for applying human skin models to in vitro skin irritation testing (Annexe 1 doc09) are based on the specifications of the two skin models that were validated during the ECVAM skin irritation validation study (SIVS), the commercially available EPISKIN and the EpiDerm test methods (Annexe 1 doc01 – doc08; doc24, doc26; ESAC 2007 see Annexe 2).

The Performance Standards describe guidance and minimum performance criteria that novel similar ('me-too') or modified test methods should fulfil so that they may be considered scientifically valid. The performance criteria include *inter alia* (a) a description of general and functional model conditions including acceptance criteria regarding the quality of individual tissues used as test system, (b) test acceptance criteria (e.g. guidance values for positive and negative control), (c) guidance regarding the test procedure and data interpretation (prediction model), (d) 20 reference chemicals that constitute a representative set of chemicals used during the full prospective validation study (3) as well as (e) performance criteria for test method reliability and predictivity.

The Performance Standards are intended as a tool to aid the evaluation, assessment and validation of novel methods on the basis of an experimental testing set of chemicals (the PS reference chemicals) that is markedly reduced in comparison with that of a full prospective validation study. According to OECD guidance document Nr. 34 on the validation and international acceptance of new or updated test methods for hazard assessment (OECD 2005), two types of test methods can be evaluated on the basis of performance standards. These are

a) Test methods that are sufficiently similar with regard to structural and functional parameters in comparison with the validated methodology ("similar methods" or "me-too methods"). The corresponding validation process is referred to as "catch-up validation".

b) Modifications of validated methods ("modified methods") which are minor enough to warrant the limited experimental assessment as outlined in the Performance Standards. The corresponding validation process is referred to as "update validation".

1.3.2. Validation of two in vitro skin irritation methods in reference to the ECVAM in vitro Skin Irritation Performance Standards

a) Test methods endorsed

The two test methods endorsed by the 29th ESAC are

1. The SkinEthic RHE model, a similar/me-too method, submitted to ECVAM as a non-ECVAM coordinated catch-up study. The test was confirmed by ECVAM as sufficiently similar with regard to its structural and functional characteristics in reference to the Performance Standards and the test method was therefore admitted as a *non-ECVAM coordinated catch-up validation study*.
2. The EpiDerm SIT model, a modification of the previously validated EpiDerm method (ESAC 2007, Annexe 2), submitted to ECVAM as a non-ECAVM coordinated update validation study. The main modification performed is the prolongation of the exposure time to the test substances from 15 ('common protocol', ECAVM SIVS) to 60 minutes, while all other essential model

parameters remained unchanged. The test method was therefore admitted by ECVAM as a *Non-ECVAM coordinated update validation study*.

It is important to note that all human reconstructed tissue models that have been validated so far for the assessment of skin irritancy potential of xenobiotics, use a post-incubation time of 42 hours. However, the assays differ with regard to the exposure time employed, i.e. the period that the epidermal surface is acutely treated with the xenobiotic. In contrast to the relatively short exposure time of 15 minutes outlined in the so-called "common protocol" of the ECVAM SIVS (Annexe 1 doc01 – doc08; doc24, doc26; ESAC 2007 see Annexe 2) the assays validated in the current context use extended exposure times: the modified EpiDerm SIT assay features, as stated above, an exposure time of 60 minutes while the SkinEthic RHE uses an exposure time of 42 minutes. The exposure times are understood to reflect the different barrier properties of the test systems and are adjusted for each test system in order to guarantee a dynamic response: the exposure time needs to be long enough to allow the development of measurable effects while being short enough to ensure that the system is not driven into saturation.

b. Submission, evaluation and peer review process

The SkinEthic RHE test method had been submitted by SkinEthic Laboratories, Nice, France on 7 April 2008. The EpiDerm SIT test method had been submitted on 23 April 2008 by the Federal Institute for Risk Assessment (BfR), Berlin, Germany.

Both test method submissions were evaluated by ECVAM on the basis of the criteria laid out in the ECVAM performance standards document (Annexe 1 doc09). In addition to the external assessment of transferability provided in both test method submissions, the transferability of the SkinEthic RHE method as well as its standard operating procedure (SOP) were independently assessed and confirmed in-house at ECVAM from March to May 2008 (test data are provided in Annexe 5). Such independent assessment by ECVAM was deemed not necessary in the case of the EpiDerm SIT method since the EpiDerm model had undergone extensive assessment during the full skin irritation validation study and since the modification of the test method was considered minor.

After ECVAM evaluation, the test method submissions and additional auxiliary material made available by ECAVM were reviewed by an ESAC Peer Review Panel and independently evaluated by this panel with regard to the ECAVM Performance Standards ((Annexe 1 doc09). The Peer Review Process was finalised on September 8, 2008.

c. Endpoints assessed by the two test methods

Both tests use the MTT test as primary endpoint. This colorimetric assay for cell viability is based on the mitochondrial reduction of the vital dye MTT [3-(4,5-Dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a purple-coloured formazan. Cell viability has been demonstrated to be a suitable parameter to extrapolate on the irritancy potential of chemicals in human reconstructed epidermis models (Annexe 1 doc06; doc26).

In addition, both the SkinEthic RHE and EpiDerm submission provided information on the secondary endpoint IL-1α (Interleukin 1 alpha). The data on IL-1α submitted in both dossiers did not demonstrate an improvement of the predictive capacity of the test methods. Therefore, for both methods, only the data for the MTT endpoint were considered with regard to the predictive capacity.

Background to the IL1 α endpoint:

As a result of the ECVAM SIVS, the IL-1α endpoint had been suggested as a potentially useful adjunct (2). IL-1α is an inflammatory mediator secreted by the non-classical pathway (Prudovsky et al.

2003, 2008). The ECVAM SIVS had concluded that IL 1α may be capable, under certain conditions, to increase the sensitivity of human reconstructed epidermis assays (ESAC 2007 Annexe 2; Annexe 1 doc06; doc26), e.g. when used in a tiered testing approach to identify false negatives of the MTT endpoint.

2. TEST SUBSTANCES USED FOR THE SKIN IRRITATION VALIDATION STUDY (SIVS)

2.1 Test substances used

The ECVAM Skin Irritation Validation Study was based on a testing set of **58 substances** that were carefully selected by applying pre-defined criteria pertaining to *inter alia* toxicological profile, physical properties, and chemical functionality (representative of innovative chemical engineering and technology) (see section 2.2).

The substances covered a spectrum of chemical functionality, physical properties and the entire range of irritancy as categorized in the rabbit Draize test. About half the test chemicals were derived from the EU New Chemicals Database (NCD), where substances frequently have complex molecular structures including multiple functional groups.

Test substance identities were derived from three sources:

1) The EU New Chemicals Database, NCD (see section 2.2.3),

2) The TSCA database (in collaboration with ICCVAM/NICEATM (see section 2.2.4)

3) The ECETOC database (see section 2.2.5)

Table 2.1 provides a summary of physical properties covered within the test set of 58 substances.

Table 2.1: *Ranges of physical properties observed within the set of test substances (n=58) used during the ECVAM Skin Irritation Validation Study.*

Physical-Chemical Property	**Range**
Molecular weight	60 - 674
LogKow	-3.4 to 11.5
Water solubility	10^{-3} to 10^{+6} mg/l
Vapour pressure	10^{-6} to 6.10^{+3} Pa
Melting point	-100 to 360 ^{o}C
Boiling point	82 to 555 ^{o}C

Table 2.2 provides the test substances with their chemical name, their CAS number, their source with respect to their substance identity, their classification according to the previous EU classification system (EU DSD; DSD=Dangerous Substance Directive, in the meantime repealed by REACH), the UN GHS system as applicable to some authorities (UN GHS*), i.e. using an additional optional category 3 and the UN GHS system as applicable to all authorities.

Table 2.2: Test substances used during the ECVAM SIVS study

Count	Code Nr.	Substance Name	CAS-Nr.	Source	Classification			dominant	
					EU DSD	UN GHS* (some authorities)	UN GHS (all authorities)	Median (Mode)	Endpoint
1	2	1-bromo-4-chlorobutane	6940-78-9	ECETOC	No	No cat.	No	0.0	B
2	6	3-diethylaminopropionitrile	02.04.5351	ECETOC	No	No cat.	No	0.0	B
3	7	3-mercaptohexanol	51755-83-0	NCD	No	No cat.	No	0.0	B
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	4166-20-5	NCD	No	No cat.	No	0.0	B
5	22	diethyl phthalate	84-66-2	ECETOC	No	No cat.	No	0.0	E
6	24	di-propylene glycol	25265-71-8	ECETOC	No	No cat.	No	0.0	E
7	25	dipropylene glycol monobutyl ether	29911-28-2	TSCA	No	No cat.	No	0.0	E
8	26	3,4-dimethyl-1H-pyrazole	2820-37-3	NCD	No	No cat.	No	0.0	B
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine-1-carboxylate	67914-69-6	NCD	No	No cat.	No	0.0	B
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	7089-59-0	NCD	No	No cat.	No	0.0	B
11	35	cyclohexadecanone	2550-52-9	NCD	No	No cat.	No	0.0	B
12	41	naphthalene acetic acid	86-87-3	TSCA	No	No cat.	No	0.0	B
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	180898-37-7	NCD	No	No cat.	No	0.0	B
14	48	2-(formylamino)-3-thiophenecarboxylic acid	43028-69-9	NCD	No	No cat.	No	0.0	B
15	53	silane A-1430	2530-87-2	TSCA	No	No cat.	No	0.0	B
16	57	triethylene glycol	112-27-6	TSCA	No	No cat.	No	0.0	B
17	9	2,6-dimethyl-4-nitrobenzeneamine	16947-63-0	NCD	No	No cat.	No	0.3	E
18	11	allyl phenoxyacetate	7493-74-5	ECETOC	No	No cat.	No	0.3	E
19	36	isopropanol	67-63-0	ECETOC	No	No cat.	No	0.3	E
20	12	2-ethylhexyl 4-aminobenzoate	26218-04-2	NCD	No	No cat.	No	0.7	E
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0319002-92-1	NCD	No	No cat.	No	0.7	E
22	5	3-chloro-4-fluoronitrobenzene	350-30-1	ECETOC	No	No cat.	No	1.0	E
23	8	4-methylthio-benzaldehyde	3446-89-7	ECETOC	No	No cat.	No	1.0	E
24	16	capryl-isostearate	209802-43-7	NCD	No	No cat.	No	1.0	E
25	39	methyl stearate	112-61-8	ECETOC	No	No cat.	No	1.0	E
26	44	phenylethylalcohol	60-12-8	ECETOC	No	No cat.	No	1.0	E

27	30	Mixture of: diethyl cis-1,4-cyclohexanedicarboxylate; and diethyl trans-1,4-cyclohexanedicarboxylate	0072903-27-6	NCD	No	No cat.	No	1.3	E
28	54	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one (CAS 224031-70-3) and 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS 224031-71-4)	224031-70-3	NCD	No	No cat.	No	1.3	E
29	10	allyl heptanoate	142-19-8	ECETOC	No	Opt Cat 3	No	1.7	E
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	128119-70-0	NCD	No	Opt Cat 3	No	1.7	E
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; and 5-endo-decylbicyclo[2.2.1]hept-2-ene	22094-85-5	NCD	No	Opt Cat 3	No	1.7	E
32	33	heptyl butyrate	5870-93-9	ECETOC	No	Opt Cat 3	No	1.7	E
33	50	2-phenylhexanenitrile	3508-98-3	NCD	No	Opt Cat 3	No	1.7	E
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	68047-07-4	NCD	R38	Opt Cat 3	No	2.0	E
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol and 56% (1'R,2R) & 40%(1'R,2S) isomer	014864-90-6	NCD	R38	Opt Cat 3	No	2.0	B
36	31	A mixture of isomers: ethyl exo-tricyclo[5.2.1.0(2,6)]decane-endo-2-carboxylate; and ethyl endo-tricyclo[5.2.1.0(2,6)]decane-exo-2-carboxylate	80657-64-3 (mix).	NCD	521	Opt Cat 3	No	2.0	O
37	34	hexyl salicylate	6259-76-3	ECETOC	R38	Opt Cat 3	No	2.0	B
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; and 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	181258-87-7 (cis), 181258-89-9 (trans)	NCD	R38	Opt Cat 3	No	2.0	B
39	46	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]decan-2-ol	122760-84-3	NCD	R38	Opt Cat 3	No	2.0	B
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	122760-85-4	NCD	R38	Opt Cat 3	No	2.0	B
41	49	isostearic acid monoisopropanolamide	152848-22-1	NCD	R38	Opt Cat 3	No	2.0	E
42	51	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS 191044-60-7) and 1-(3-isopropylphenyl)-1-phenylethane (CAS 191044-59-4) and 1-(4-isopropylphenyl)-1-phenylethane (CAS 2320-06-1)	52783-21-8 (mix.)	NCD	R38	Opt Cat 3	No	2.0	E
43	55	terpinyl acetate	80-26-2	ECETOC	R38	Opt Cat 3	No	2.0	B
44	58	tri-isobutyl phosphate	126-71-6	TSCA	R38	Opt Cat 3	No	2.0	E
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	not allocated	NCD	R38	Opt Cat 3	No	2.0	E
46	4	1-decanol	112-30-1	ECETOC	R38	Cat. 2	Cat. 2	2.3	E
47	20	cyclamen aldehyde	103-95-7	ECETOC	R38	Cat. 2	Cat. 2	2.3	O
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	86604-75-3	NCD	R38	Cat. 2	Cat. 2	2.7	B
49	3	1-bromohexane	111-25-1	ECETOC	R38	Cat. 2	Cat. 2	2.7	E
50	15	a-terpineol	98-55-5	ECETOC	R38	Cat. 2	Cat. 2	2.7	O

51	**45**	**(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol**	**107898-54-4**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**2.7**	**E**
52	**18**	**butyl methacrylate**	**97-88-1**	**TSCA**	**R38**	**Cat. 2**	**Cat. 2**	**3.0**	**E**
53	**23**	**di-n-propyl disulphide**	**629-19-6**	**ECETOC**	**R38**	**Cat. 2**	**Cat. 2**	**3.0**	**E**
54	**37**	**[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)**	**not allocated**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**3.0**	**E**
55	**40**	**1-methyl-3-phenyl-1-piperazine**	**5271-27-2**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**3.3**	**E**
56	**56**	**benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name from company)***	**7340-90-1**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**3.3**	**O**
57	**27**	**2-isopropyl-2-isobutyl-1,3-dimethoxypropane**	**129228-21-3**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**4.0**	**E**
58	**59**	**(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol**	**125474-34-2**	**NCD**	**R38**	**Cat. 2**	**Cat. 2**	**4.0**	**E**

E = Erythema, O = Oedema, B = Both.

2.2 Test substance selection ("chemicals selection")

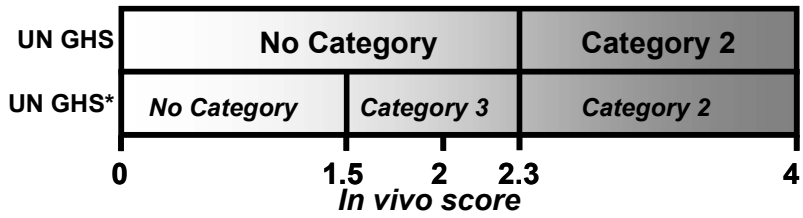
2.2.1 Background

a) The chemicals selection in the light of the objectives of the SIVS

The **primary objective** of SIVS was to evaluate whether the EpiDerm and EPISKIN assays could reliably distinguish skin irritants (classified with risk phrase R38) from non-irritants (no label), according to the EU system in force under *Directive 67/548/EEC* (EC 2001), the so-called "Dangerous Substance Directive"(DSD). For statistical reasons, a sufficient number of test substances are required to test either a classification system of either two or three categories. The statistical background is briefly summarised in section 2.2.2 (for details see Annexe 1 doc06).

A **secondary objective** was to make a retrospective analysis of the data, to assess whether the *in vitro* tests would be able to discriminate between GHS strong irritant (category 2), mild irritant (category 3) and non-irritant (no category) chemicals. Although primarily a one category scheme (see paragraph 3.2.2.5.1 of the GHS document, United Nations 2008), UN GHS allows the use of an optional additional category 3 for "authorities that wish to have more than one irritant category" (paragraph 3.2.2.5.1 subpoint c, United Nations 2008). Thus, UN GHS foresees two compatible classification schemes, one on the basis of a single irritant category only (2.3 ≥ *in vivo* score ≤ 4.0) and another scheme on the basis of this category plus a mild category ranging from *in vivo* scores of 1.5 to 2.3 (1.5 ≥ *in vivo* score < 2.3). The two classification systems compliant with the provisions UN GHS are illustrated schematically in **Figure 2.1**.

Figure 2.1: *UN GHS allows the use of maximally two irritant categories (Category 2 and Category 3). While Category 2 is applicable to all authorities, Category 3 is an "opt-in" additional category for authorities that wish to have more than one category. The two categories differ mainly in the severity of the skin reactions). Thus, depending on the needs of the authorities, two UN GHS-compliant systems result. These are abbreviated in this document as follows: "UN GHS" using only Category 2 and "UN GHS*" using Category 2 and the optional Category 3.*



b) Definitions in the context of the chemicals selection

- ***'Substance':***
  refers to a reaction product obtained from a chemical synthesis, which may comprise more than one molecular component (substance components), and frequently includes by-product impurities. By convention, substance components are defined as molecules present at >10%. Impurities are defined as molecules present at <10%.

- ***'Single component substance'***
  An individual molecule present at >80% defines a single component substance, identified as that one molecule name only. Individual molecules present in the range >10% to <80% define components of a reaction mixture substance, identified with all component molecule names. Typically, reaction mixtures would comprise isomers or generically related species as components. Impurities are not specifically identified as part of the substance name unless significant contribution is made to the hazard classification.

- ***'Preparation'***
  The term preparation refers to a formulated blend of substances, integrated in measured proportions appropriate for storage, transport, marketing, use application, etc. Regulatory hazard classification of preparations is normally derived from toxicological data on individual ingredients, with application of rule based estimation according to substance proportions.

- ***'Formulation'***
  Synonym to 'preparation'. The term 'formulation' is often used in the context of cosmetic use and application of substances and preparations thereof.

2.2.2 Summary of the chemicals selection process

a) Number of chemicals used in the SIVS

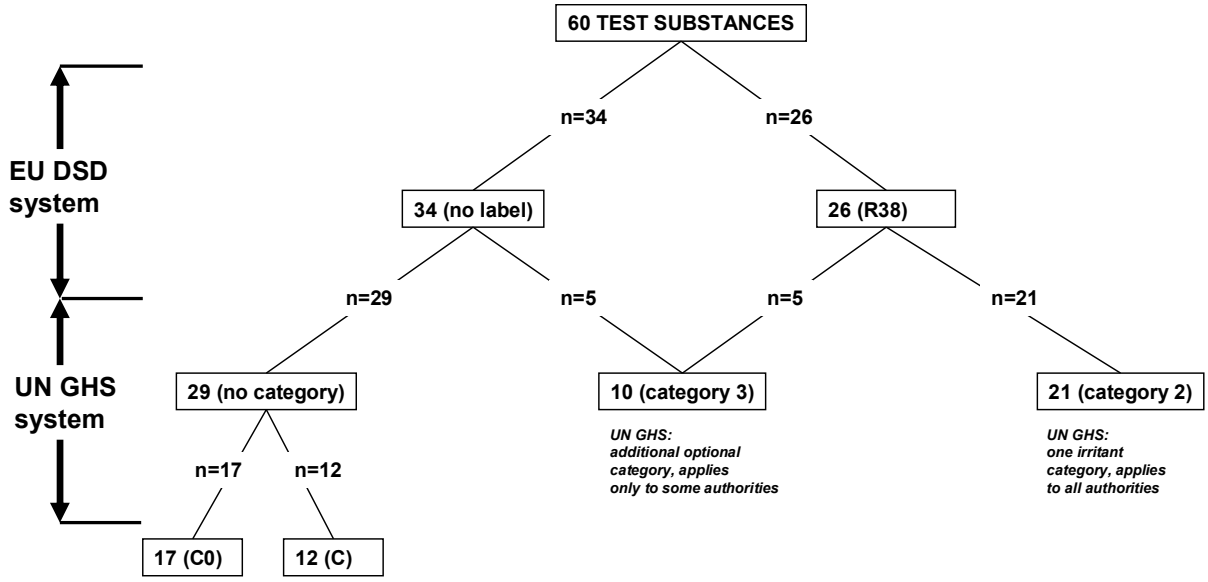
The requisite number of chemicals for *in vitro* testing was determined according to statistical sample size calculation, considering sensitivity and specificity relative to a dichotomous classification system (one irritant category versus a class of non-classified substances), i.e. the previous EU classification system (EU DSD) as the principal parameters of interest. For simplicity, independence of these two parameters (and independence of classification of a given chemical from the classification of another chemical) was assumed. Considering the experience of the laboratories with the assays, and setting a minimum requirement in terms of predictive capacity, attainment of specificity and sensitivity to at least 75% was presumed as acceptance criterion. Based on these assumptions, it was estimated how large the sample size for a binomial proportion had to be, in order to have a lower limit of a one-sided confidence interval significantly larger than 0.5, with a significance level of 0.05 and with a power of at least 0.75, as generalised by Flahault et al. By using the software package, S-Plus 6.1 (Insightful, USA) it was determined that 26 chemicals, respective each of irritants and non-irritants, would be necessary.

On this basis, the project management set a total of 60 chemicals to be selected. The number of R38 skin irritants was set to 26 chemicals (note that upon completion of the SIVS this number was reduced to 25, since the identity of one of the substances could not be disclosed. The substance was consequently excluded from the analysis).

It is noteworthy that out of the 26 irritant chemicals, 12 substances had an in vivo score of 2.0 (cut-off score between no label and R38), while 13 covered the further range from 2.3 to 4.0. This number of substances was deemed sufficient to assess the accuracy and reliability of the methods for the irritant range, while a high number of borderline substances (score 2.0) was deliberately selected to challenge the test methods and be able to analyse the accuracy of their predictions in this difficult band of substances with scores at the cut-off line.

An excess of non-irritants was conscientiously decided in acknowledgement of the relatively high proportion of chemicals with oedema and erythema scores of 0, i.e. the high prevalence of non-irritant substances (Hoffmann et al. 2005). With consideration to the secondary objective, requiring representation of the (maximally) three UN GHS compliant categories, a selection distribution with respect to EU and GHS classifications was proposed according to Figure 2.2.

Figure 2.2. *Target distribution of SIVS reference chemicals with respect to EU DSD and UN GHS* classification systems (as applicable to only some authorities). 'C0' represents chemicals with oedema and erythema scores of 0, 'C-'represents GHS non-classified chemicals with oedema and/or erythema scores different from 0.*



b) Principal selection criteria

(1) Availability of *in vivo* reference data of high quality

A principal criterion for selection of chemicals was the availability of supporting quality assured *in vivo* data, with which to correlate the *in vitro* data.

(2) "New chemistry"

Another important criterion for selection, aiming to challenge the *in vitro* test models and thereby add credibility to the validation, was to seek commercial industrial chemicals representative of current innovative engineering technology, including multi-functional molecular class and diverse use applications. The first source of chemicals screened was the New Chemicals Database (NCD) managed by the former European Chemicals Bureau (ECB, a partner unit of ECVAM at the European Commission Joint Research Centre). NCD was the central archive within the EU notification scheme for 'new' commercial chemicals, defined as substances introduced to the EU industrial market after 1981. Files registered in NCD were subject to regulatory review and approval. Integral to comprehensive hazard assessment obligations, skin irritation testing was requisite according to the standard *in vivo* method (OECD TG 404; Method B.4 in Annex V of *Directive 67/548/EEC*). NCD therefore offered a unique and operational source for the selection of candidate chemicals, supported by reviewed, standardised and quality compliant *in vivo* data.

However, it should be noted that NCD notification files are confidential, with access only permitted by appointed personnel from EU regulatory agencies (Competent Authorities) and the European Commission. Release of data on individual substances (e.g. for independent projects, in this case, SIVS) would therefore require prior consent of respective proprietors. Moreover, notified chemicals are typically produced according to particular industrial applications with market supply oriented to use specific syntheses or formulation processes. Therefore, notified substances are not normally available through regular laboratory supply catalogues, and acquisition of material would again require cooperation of manufacturers/notifiers to make exception to normal marketing practice by providing samples for *in vitro* study.

Therefore, additional 'existing' candidate chemicals, which were readily available from major manufacturing and/or distribution sources, were identified to supplement to the NCD chemicals: These supplementary chemicals with in vivo data of appropriate quality were from a) the TSCA (Toxic Substance Control Act) database maintained by the US EPA; b) an ICCVAM public call for the submission of dermal irritancy chemicals and protocol information/ test data; and c) the ECETOC database, excluding those chemicals used in the previous optimisation and prevalidation phases.

Analysis of prevalence among ~3100 chemicals surveyed from NCD and ECETOC found 8% were skin irritant to rabbit (i.e., EU classification R38) and 64% with zero oedema and erythema irritation scores (Hoffmann et al. 2005).

2.2.3 Selection of 'new' (notified) chemicals from NCD

When the SIVS chemical selection process started, the EU New Chemicals Database (NCD) contained 3278 chemicals with test data on skin irritation and skin corrosion. Reducing this set by subtracting corrosive substances led to a pool of 3121 substances. Pre-defined selection criteria were applied to this pool of chemicals in order to establish a testing set that would meet the prerequisites of test chemicals for the purposes of a validation study. The criteria were applied in four steps (described in detail in the following sections):

1. Primary extraction (section a)
2. Secondary reduction (section b)
3. Selection refinement (section c)
4. Substances obtained from suppliers (section d)

By applying these step-wise approach the initial set of 3121 substances was reduced to only 126 substances that met all of the criteria. Of those only 37 substances were still available through the original notifying suppliers.

Table 2.3: *Extraction of appropriate test substances form the EU New Chemical database. The table shows how, by application of various exclusion, extraction, reduction and refinement criteria and, considering commercial availability, test substances with properties appropriate for purposes of a validation study were extracted. Note that only 37 out of 3278 chemicals met all criteria (including availability from original suppliers). This demonstrates that identification of appropriate chemicals represents a major hurdle for a validation study, and that the selection of substances that are useable is in fact very limited.*

	EU R38		EU no label		Total Number
	GHS cat. 2	GHS cat. 3	GHS cat. 3	GHS no cat.	
Starting Pool NCD including corrosives					**3278**
Starting Pool NCD without corrosives	**246**		**2875**		**3121**
Primary extraction (section a)	132		1175		1307
Secondary reduction (section b)	28	59	27	218	332
Selection refinement (section c)	11	21	7	87	126
NCD selected substances obtained from suppliers (section d)	**7**	**11**	**4**	**15**	**37**

a) Primary extraction of NCD chemicals

At the time of the chemicals selection, in the spring of 2003, NCD contained approximately 5600 notifications, covering about 3500 substances. The following exclusion criteria were applied in a **primary survey and extraction of data**:

1. Substances notified before 1995 were initially excluded, focusing the search on notifications from the previous decade, for which complete electronic data files would be assured, and where contacting companies for the supply of substance sample material would be facilitated.

2. For multiple dossiers concerning the same substance, repeat notifications were excluded, and only data from the file leader, usually the original notifier, were extracted.

3. Substances marketed at quantities below 0.1 tonnes/year, for which skin irritation testing was not a regulatory requirement, were excluded.

4. Skin corrosives were excluded, as the primary focus was on skin irritation.

5. Adsorbents, gases and vapours were excluded for technical reasons linked to the test protocols being validated.

Applying these exclusion criteria, the NCD survey yielded 1307 substances, comprising 132 skin irritants (R38) and 1175 non-irritants (no label), based on skin irritant classifications on file registered by EU Competent Authorities. The following information on these substances was then extracted:

1. substance identification and molecular structure;
2. physical state (solid/liquid);
3. purity (typical %, lower limit %, upper limit %);
4. skin irritation data (*in vivo* erythema and oedema scores);
5. classification and labelling;
6. whether a mixture (Y/N);
7. molecular weight (MW), including components in mixtures;
8. octanol–water partition coefficient (log K_{ow});
9. water solubility;
10. vapour pressure;
11. melting point and boiling point;
12. desired effect and use categories; and
13. name(s) of producer and/or notifier (including country of origin).

b) Secondary reduction of NCD chemicals

A secondary data reduction applied the following quality criteria on the extracted physico–chemical properties:

1. Substances with no known purity, or with a typical purity of less than 94%, were disregarded.

2. According to regulatory definitions, single substances can comprise reaction mixtures of unseparated components, such as isomers or generically similar molecules. Substances with more than three components (or more than four components for isomeric mixtures) were excluded.

3. Complex mixtures, substances with unidentified components, and substances with uncertain component proportions, were excluded.

4. Substances with no known MW, with a MW over 1000, or with a range of MWs (e.g. polymers), were excluded.

No exclusion criteria were applied on the basis of water solubility, log K_{ow}, vapour pressure, boiling point or melting point, in order to incorporate variability in these physical-chemical characteristics. In addition, no exclusion criteria were applied on the basis of other topical irritation, namely, skin sensitisation and eye irritation, again to allow scope for variability.

Following application of the secondary elimination criteria, 845 candidate chemicals remained from the primary list of 1307, including 87 irritants and 758 no-label chemicals. The *in vivo* data (erythema and oedema scores) for the short-listed substances were reviewed, with irritant classifications being assigned according to the GHS classification scheme. Due to an excessive number of GHS non-irritant substances (731), a pragmatic refinement gave preference to irritant substances notified by the same suppliers, and to substances indicated for use as cosmetic ingredients. Based on this refinement, a total of 218 non-irritants were short-listed.

c) Refinement of the selection of NCD chemicals

The short-listed skin irritants and non-irritants were then further screened for properties which would cause practical difficulties in testing, as well as for any classification inconsistencies. The following refinement criteria were applied:

1. Particularly hazardous chemicals, such as carcinogens and explosives, were eliminated for safety reasons.

2. Chemicals likely to present testing difficulties, such as those with hydrolysing properties, polymerising tendencies, or samples available only as preparations, were excluded.

3. Chemicals no longer in production (reported by a regulatory authority) were excluded.

4. Chemicals having classifications inconsistent with the Draize test scores were disregarded. These comprise chemicals classified on the basis of a non-standard method, read-across, or persistent effects. Regarding this latter criterion, a supplementary study was initially proposed where the lead laboratories would also assay chemicals classified on the basis of persistence. However, of all the chemicals screened, only three substances were found to be classified as R38 on the basis of persistence of effects. In addition, only one could be obtained from the contacted suppliers, and anyway, insufficient information was available to allow its inclusion in the study. As a consequence, the proposed parallel study could not take place.

Overall, the described selection criteria resulted in short-listing of 27 R38 and 94 no-label chemicals. However, as the number of skin irritants barely fulfilled the minimum sample size required (and in anticipation of unattainable cooperation of manufacturers/suppliers) it was decided to extend the scope of the primary NCD survey to include R38 substances notified prior to 1995. Primary extraction yielded a further 54 chemicals, of which five R38 substances met the secondary reduction and selection refinement criteria.

A total of 126 substances were finally short-listed from NCD as eligible test materials for SIVS, comprising 32 skin irritants and 94 non-irritants. For quality control, it was reconfirmed that the assigned classifications and labelling (R38 or no label) proposed by the Competent Authorities and/or those published in Annex I of *Directive 67/548/EEC*, could be derived from the rabbit Draize scores for these substances.

d) Suppliers contact and confidentiality issues

A total of 58 notifiers and/or producers were identified for supplying the 126 selected substances. In cases where contact addresses were obsolete, updated information was sought, by invoking assistance of the European Chemical Industry Council (CEFIC) and the European Federation for Cosmetic Ingredients (EFfCI). Subsequently, 47 producers and/or notifiers were successfully contacted for the supply of 115 of the 126 selected substances, and sample materials were requested. Responses were eventually received from 30 companies, of which 25 were able to cooperate by providing a total of 37 test samples, comprising 18 skin irritants and 19 non-irritants.

During the course of the study, collaborating companies were requested to release the identities (IUPAC names, CAS numbers, and structural formulae) of the selected notified chemicals, which would otherwise have been restricted as confidential proprietary information. Of the 25 suppliers, 24 agreed to release substance description, covering 35 of the 37 substances. Furthermore, agreement to release the skin irritation classifications was confirmed with these suppliers. However, it was agreed that the individual suppliers would remain anonymous, and would not be associated with any specific substances, nor would the corresponding Draize scores registered for the NCD substances be disclosed. Permission was not obtained for the disclosure of the identities of the two remaining substances. Consequently, the *in vitro* results obtained for these two substances were not considered in the final evaluation of the SIVS.

Due to the shortfall in availability of the NCD chemicals and the need to include chemicals which were readily available from major manufacturing and/or distribution sources, supplementary 'existing' chemicals were also selected, through recourse to other databases.

2.2.4 Collaboration with NICEATM-ICCVAM

NICEATM-ICCVAM, in collaboration with DCIWG, provided two lists of candidate chemicals, based on the following sources: data submitted to the EPA in accordance with TSCA, and data submitted in response to a public call for Draize dermal irritation data.

In summary, 6 substances from ICCVAM-NICEATM files qualified for inclusion in SIVS, supported by Draize rabbit scores compliant with standard protocols, and fulfilling the secondary reduction and selection refinement criteria, as described above. The 6 substances provided two skin irritants (R38) and four non-irritants (no label, according to the EU system).

2.2.5 ECETOC Database

The ECETOC database for skin irritation/skin corrosion contains a total of 129 chemicals, for which detailed *in vivo* data from dermal irritation studies are available. From application of the secondary reduction and selection refinement criteria to 34 eligible substances that had not previously been tested in the prevalidation and protocol optimisation phases of SIVS, 14 chemicals were shortlisted. These were supplemented with 5 chemicals which had been used in previous protocol optimisation and prevalidation phases, in order to complete the 60 chemicals required for SIVS. The 19 ECETOC chemicals included 7 skin irritants (R38) and 12 non-irritants (no label, according to the EU system).

2.2.6 Chemical classification of the test substances

Characterisation of chemical class among the set of substances selected for SIVS would provide a basis for defining the limitations in terms of chemical applicability domain for the *in-vitro* methods. However, occurrence of multiple functional groups, evident within single molecular species, presents scope for alternative chemical class description, precluding systematic allocation of a definitive category to each individual substance. Indeed, division of the 60 SIVS chemicals according to molecular genus, based on review perspectives of organisations affiliated to the project (*viz*. ECB, BfR, Syngenta, and NICEATM) yielded alternative classes, with no general consensus on distribution. For illustration, chemical class distribution according to MeSH analysis by NICEATM (Table 2.4) yielded ~100 categories for the 60 substances, reflecting the multi-functional nature of the molecular structures involved.

Table 2.4 *MeSH* analysis of SIVS chemicals.*

Alcohols	14		Ketones	4
Aldehydes	3		Lactose	1
Amides	2		Nitriles	2
Amines	8		Nitro compounds	2
Cyclic	1		Organophosphates	1
Carboxylic acids	2		Organosilicon compound	1
Esters	19		Phenol	1
Ethers	13		Polycyclic	6
Halogenated	2 (+3)		Salts	(1)
Heterocyclic	9		Sulphur compounds	6
Hydrocarbons	3 (+3)		Zinc	1

**MeSH (Medical Subject Headings) used by the US National Library of Medicine, is a hierarchical thesaurus for indexing documents etc.*

Moreover, examination of SIVS misclassifications *in vitro* (i.e., false positives/negatives with respect to accepted classifications *in vivo*) found neither any systematic influence nor any mechanistic explanation for the disparities, perceptible from review of the following (Zuang et al. 2007):
1) range of *in vivo* data;
2) structure-activity relationships (Q)SARs;
3) physical-chemical properties;
4) artefact of eye irritation or skin sensitisation;
5) observed experimental anomalies.
Apparently, skin irritation is an empirical phenomenon without characteristic mechanism *in vivo*, and therefore without evidence for limitation of applicability domain *in vitro*.

2.2.7 Summary

In total, approximately 3500 'new' (EU notified) chemicals registered in NCD, and 1600 'existing' chemicals recorded in alternative databases, such as the TSCA, CIR and ECETOC databases, were screened. Only a very limited number fulfilled the selection criteria established prior to the selection process pertaining to the SIVS, where the project management set a total of 60 chemicals required for *in vitro* assay.

35 originated from NCD and 25 from the supplementary databases. Due to unresolved confidentiality issues, identities of two notified chemicals (one R38 and one no-label) could not be disclosed. As a consequence, these two substances were omitted from the validation analysis, effectively leaving 33 NCD chemicals, and reducing the overall total reported from 60 to 58.

Table 2.5 shows the distribution of the 58 chemicals respective of the EU DSD and the UN GHS compliant classification systems, which prior to omission of the 2 confidential chemicals (parentheses in **Table 2.5**) had included 26 EU irritants (labelled R38) and 34 EU non-irritants (not labelled).

Table 2.5: *Distribution of test substances according to the various classification categories and with respect to their origin from the three sources: EU new chemicals database (NCD), the ECETOC database and the TSCA database.*

Source	**R38 (skin irritants)**		**No label (non irritants)**		**Totals**
	UN GHS cat. 2	UN GHS* cat. 3 *(no cat. Under UN GHS)*	UN GHS* cat. 3 *(no cat. Under UN GHS)*	UN GHS no cat.	
NCD	7	9 (10)	3	14 (15)	33 (35)
ECETOC	5	2	2	10	19
TSCA+CIR	1	1	0	4	6
Totals	**25 (26)**		**33 (34)**		**58 (60)**

The original distribution of 60 chemicals fulfilled the primary selection objective of division between EU irritants and non-irritants. With reference to the secondary SIVS objective, the selected chemicals also presented a balanced distribution among the three possible GHS categories, allowing for a *post hoc* evaluation. However, there was a shortfall of GHS category 2 irritants, where only 13 were found, rather than 21 statistically recommended (Hoffmann et al. 2005) apparently related to low prevalence of GHS category 2 chemicals in the databases surveyed.

In compensation, 17 GHS category 3 chemicals were obtained, in excess of a statistical recommendation of 10 (Hoffmann et al. 2005) reflecting higher prevalence of *in vivo* observations near to the EU classification threshold of 2. Figure 2.3 shows the frequency distribution (histogram) of irritant response in vivo among the 58 chemicals, characterised by dominant median Draize score, where the relatively high prevalence of zero values and values of 2.0 is evident. Figure 2.4 shows the chemicals as a scatter plot.

Note the high number of chemicals with scores of 0 (reflecting the high prevalence) and with score of 2.0 (reflecting the arbitrary decision to challenge the test methods at the cut-off score).

Figure 2.3: *Histogram (frequency distribution) of the irritant response in vivo among the 58 chemicals according to the dominant median Draize score. The histogram clearly shows that borderline substances (with a score of 2.0 = cut-off for the categorical decision) are grossly overrepresented in order to allow an analysis of the accuracy of the models at this challenging zone.*

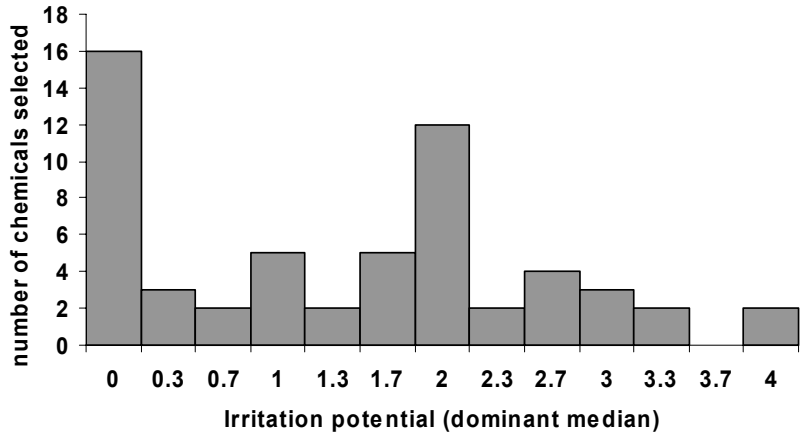
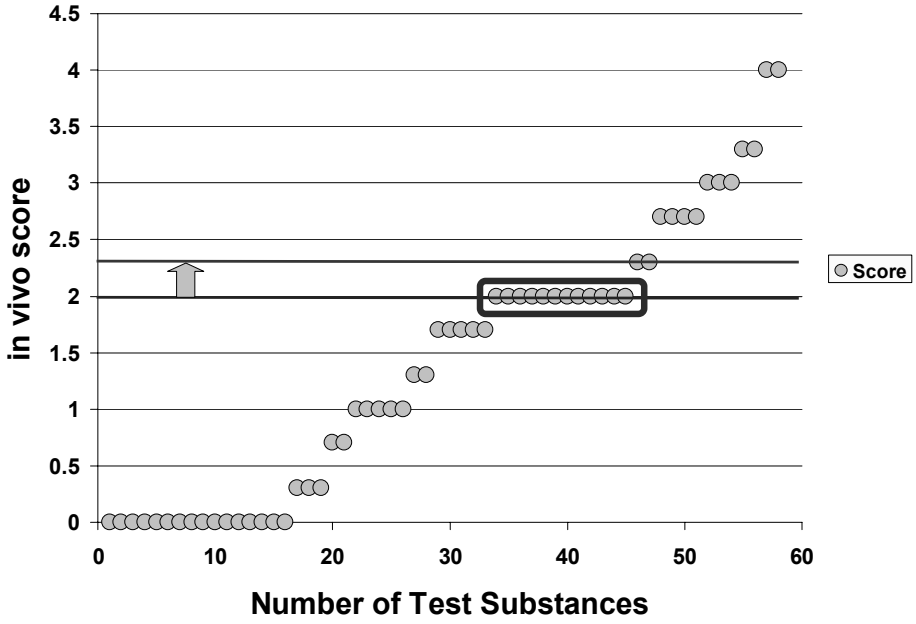


Figure 2.4: *Scatter plot of the in vivo scores of the test substances used in the SIVS. The substance scores are distributed from 0 to 4.0. Evidently substances with a score of 0 and those with a score of 2.0 are overrepresented. The overrepresentation of score 0 substances is in agreement with the high prevalence of such substances in representative populations (prevalence may be as low as 7.5% [EU DSD] or about 3% [UN GHS]). Score 2.0 substances however were overrepresented (red box) to allow assessment of the predictive accuracy of the test methods at the borderline zone. The cut-offs for EU DSD (purple) and UN GHS (red) are indicated.*



2.3 Analysis of the substances tested during validation and optimisation with respect to a list of 'common irritants' from 1981

The previous section has dealt with a possible classification of chemical substances used during the SIVS with respect to their grouping into chemical classes based on their chemical structure using the MeSH classification system.

This section now addresses the issue of categorisation from the other direction: to which extent were the chemicals used in the validation and optimization studies representative for chemical classes/groups which by experience are known to contain irritants?

The basis for this analysis was the publication "Manual of contact dermatitis" (edited by S. Fregert, published in 1981), that provides a section (nr. 8) in which so-called "common irritants" are described[3]. While the materials listed in this book may be based on practical experience, this list should not be taken without the following caveats:

1) This is only one of many possible lists of "common irritants" and should not be understood to constitute a scientific consensus agreement with respect to what constitutes 'common irritants' and what not.
2) The categorisation of the Fregert publication is rather superficial and does not provide a fine resolution with respect to chemical classes/functional groups or materials (in 8.10 for example, entire plants or plant parts are being listed).
3) It is important to note that chemical substances of contemporary chemistry contain very often several different functional groups which make their categorisation acc. to chemical structure / functionality very difficult. Inversely, it is probably impossible to extrapolate from chemical structure of substances of a contemporary chemistry to their irritancy potential.
4) Fregert's list of "common irritants" is not backed by empirical evidence at the level of single compounds and appropriate references to testing data/publications It is therefore rather difficult to judge how and why these categories were listed as 'common irritants'.
5) It should be noted that not necessarily all substances that fall into the listed categories are automatically irritants. What Fregert's list of common irritants provides is a list of chemical categories, use groups or even materials that frequently contain irritant substances or may – under specific circumstances – cause irritation (remarkably water is listed as entry Nr. 1). Although some suggested classes, such as in section 8.7 organic solvents, may be regarded as irritants due to their mechanism of action, it does by no means follow, that ALL substances that fall into one of the categories listed by Fregert are automatically irritants. For example, the Fregert publication states in section 8.5 that "among saturated free fatty acids, particularly C_8 and C_{12} are irritating". However, dodecanoic acid (synonym lauric acid), used during the optimisation studies is NOT an irritant (in vivo score 0.3), although being a C_{12} fatty acid. This contradicts the rather simplistic generalisation of the list of "common irritants" provided in the Fregert publication.

[3] Fregert S (1981) Manual of Contact Dermatitis. Second Edition. Munksgaard, Copenhagen.

2.3.1. Substances not tested during validation and optimisation that fall into the "common irritant" categories by Fregert, published in 1981)

Out of the categories listed by Fregert, the following chemical categories were not tested: aliphatic amines, aromatic organic solvents, quartenary ammonium compounds, metal compounds and oxidants *e.g.* organic peroxides. It should be noted that organic peroxides (also listed) are generally corrosive substances and would thus be identified by other means of testing (e.g. within the testing strategy of TG 404). However, since the draft test guideline is for both substances and mixtures, testing information on mixtures containing organic peroxides or on appropriately diluted organic peroxides may be useful and is at present not available.
Apart from these 4 chemical categories, enzymes (also listed as common irritants) were not tested either.

Substances likely to be used as ingredients for cosmetics were cross-checked in the COSING database of the European Commission http://ec.europa.eu/enterprise/cosmetics/cosing/. The results of this analysis are shown in blue ("COSING") in table 2.6.

In conclusion, the following are classes/groups of chemicals, which may contain irritants, and which have not been included in the validation or optimization phases:

- Enzymes
- Alkalis, including aliphatic amines
- Organic solvents, aromatic
- Oxidants (e.g. organic peroxides)
- Quarternary ammonium compounds
- Metal compounds

2.3.2 Plant ingredients

Moreover, Fregert lists plants as possible sources of irritant substances: *"orange peel and hyacinth and tulip bulbs contain irritants, as do pineapple juice, cucumbers, asparagus, mustard, barley and corn, spurge, pasque flower, wind flower, rice, bamboo" (Paragraph 8.10).* Notably, the publication by Fregert (presumably due to the publication date 1981) does not provide any single plant ingredients identified as irritant.

Notably, during both the validation (A) and optimisation studies (B) several substances naturally occurring in plants (plant ingredients) or derived from plant ingredients were tested (see table **2.7**, next page). Under EU DSD 10 of these are regarded irritant, while under UN GHS – due to the shift in cut-off value – only 3 of them are considered irritant. Many of these are important ingredients in the fragrance/cosmetics industry.

Table 2.7 *Irritant plant ingredients tested during validation and optimisation studies. Also non-irritants tested are listed for completeness. Substances are listed in order of decreasing irritancy. Irritant classes/categories in pink; no label/category in green.*

Nr.	Substance tested	Possible plant sources	Score	EU DSD	UN GHS
1	Methyl palmitate (B)	In essential oils of many plants, e.g. palm, lavender, sage, star fruit etc.	3	R 38	Cat. 2
2	Alpha terpineol (A + B)	A monoterpene alcohol that can be found in pine oil, cajuput oil (from *Melaleuca leucadendra* and probably other *Melaleuca* species) and petitgrain oil (from bitter oranges).	2.7	R 38	Cat. 2
3	Cyclamen aldehyde (A)	Toxic alkaloid of the *primulaceae* family	2.3	R 38	Cat. 2
4	Terpinyl acetate (A)	Essential oils of e.g. blood organge, bergamot, basil	2	R 38	Opt. cat. 3
5	dl Citronellol (B)	In essential oils of e.g. lemon grass, orange flower, lavender	2	R 38	Opt. cat. 3
6	Cinnamaldehyde (A + B)	In the bark of the cinnamon species	2	R 38	Opt. cat. 3
7	d-Limonene (B)	Cyclic terpene found e.g. in orange peel	2	R 38	Opt. cat. 3
8	Linalol (B)	In essential oils of lavender, bergamot, rose wood and lily of the valley	2	R 38	Opt. cat. 3
9	Linalyl acetate (B)	In essential oils of bergamot and lavender	2	R 38	Opt. cat. 3
10	Eugenol (B)	In essential oils of clove, nutmeg, cinnamon, basil, bay	2	R 38	Opt. cat. 3
11	Isopropyl palmitate (B)	Derived from e.g. palm oil	1	No	No cat.
12	Soap from 20/80 coconut oil/tallow (B)	From coconuts	1	No	No cat.
13	Hydroxycitronellal (B)	In essential oils of e.g. lily of the valley	1	No	No cat.

2.3.3. Inorganic substances

During the optimisation phase the following inorganic substances were tested:

Nr.	Substance tested	Score	EU DSD	UN GHS
1	Sodium bicarbonate (B)	0	No	No cat.
2	Sodium bisulphite (B)	1	No	No cat.
3	Potassium hydroxide, 5% (B)	3	R 38	Cat. 2

Irritant classes/categories in pink; no label/category in green.

3. PERFORMANCE OF THE THREE ECVAM-VALIDATED SKIN IRRITATION METHODS

This section describes the performance of the three validated methods under

1) the previous EU classification system (EU DSD) and
2) the UN GHS system as applicable to all authorities (i.e. using one single irritant category) and as implemented in the EU through the CLP regulation.

3.1 Summary of accuracy values under EU DSD and UN GHS

Table 3.1. *Accuracy values for the three ECVAM-validated stand-alone skin irritation in vitro test methods under EU DSD and UN GHS classification system based on the MODE of individual laboratory predictions ("final decision over all participating laboratories"). Due to the inversion of the categorical assignment of substances with in vivo scores form 2.0 to 2.3 there is also an inversion of the specificity and sensitivity values. Since under UN GHS substances with scores only from 2.3 on are regarded irritant, the sensitivity of the test system is increased, while the specificity is decreased. Note that all predictions from 0 to 2.0 and from 2.3 to 4.0 are not affected by the threshold shift from 2.0 to 2.3 (under UN GHS) with regard to the cut-off value between irritants and non-irritants.*

	EpiSkin (58 chemicals[1])		**EpiSkin (20 reference chemicals[3])**		**Modified EpiDerm (20 reference chemicals[3])**		**SkinEthic (20 reference chemicals[3])**	
	EU DSD	**UN GHS**	EU DSD	**UN GHS**	EU DSD	**UN GHS**	EU DSD	**UN GHS**
Sensitivity (%) [2]	72.0	**84.6**	70.0	**85.7**	80	**85.7**	90	**100**
Specificity (%) [2]	81.8	**71.1**	80.0	**76.9**	80	**69.2**	80	**69.2**
Overall Accuracy (%) [2]	77.6	**74.1**	75.0	**80**	80	**75**	85	**80**

[1]) The test substances from the ECVAM Skin Irritation Validation Study (SIVS) conducted from 2003 to 2007.
[2]) Based on the median (or mode) of the individual laboratory predictions.
[3]) Original 20 RC from the ECVAM Performance Standards, May 2007

Table 3.2. *Synoptic view of the contingency tables of predictions under EU DSD (a,c,e,g) and UN GHS (b,d,e,h) obtained during the two sets of validation studies, the SIVS (a-d) and two equivalence validation studies based on Performance Standards (PS) (e-h). Calculations are based on the final decision (mode) over all participating laboratories.*

EpiSkin test method (SIVS)

a **EU DSD**

		in vitro I	NI	Σ
in vivo	I	18	7	25
	NI	6	27	33
	Σ	24	34	***58***

Sensitivity (%)	72.0
Specificity (%)	81.8
Accuracy (%)	77.6

b **UN GHS**

		in vitro I	NI	Σ
in vivo	I	11	2	13
	NI	13	32	45
	Σ	24	34	***58***

Sensitivity (%)	84.6
Specificity (%)	71.1
Accuracy (%)	74.1

original EpiDerm test method (SIVS)

c **EU DSD**

		in vitro I	NI	Σ
in vivo	I	15	10	25
	NI	4	29	33
	Σ	19	39	***58***

Sensitivity (%)	60.0
Specificity (%)	87.9
Accuracy (%)	75.9

d **UN GHS**

		in vitro I	NI	Σ
in vivo	I	8	5	13
	NI	11	34	45
	Σ	19	39	***58***

Sensitivity (%)	61.5
Specificity (%)	75.6
Accuracy (%)	72.4

modified EpiDerm test method (PS based study)

e **EU DSD**

		in vitro I	NI	Σ
in vivo	I	8	2	10
	NI	2	8	10
	Σ	10	10	***20***

Sensitivity (%)	80.0
Specificity (%)	80.0
Accuracy (%)	80.0

f **UN GHS**

		in vitro I	NI	Σ
in vivo	I	6	1	7
	NI	4	9	13
	Σ	10	10	***20***

	85.7
	69.2
	75.0

SkinEthic test method (PS based study)

g **EU DSD**

		in vitro I	NI	Σ
in vivo	I	9	1	10
	NI	2	8	10
	Σ	11	9	***20***

Sensitivity (%)	90.0
Specificity (%)	80.0
Accuracy (%)	85.0

h **UN GHS**

		in vitro I	NI	Σ
in vivo	I	7	0	7
	NI	4	9	13
	Σ	11	9	***20***

	100.0
	69.2
	80.0

3.2 Detailed information of the performance under the previous EU classification system and the UN GHS system

3.2.1 Performance of the EpiSkin Test Method under EU DSD versus UN GHS system during the SIVS

This section provides all relevant data and test outcomes for the EpiSkin test method during the SIVS. **Table 3.3** provides the contingency tables for the EpiSkin test method upon completion of the SIVS based on the EU DSD system (3.3 a) and the UN GHS system (3.3 b). **Table 3.4** presents the individual test results from all laboratories for all 58 chemicals. **Table 3.5** provides the test outcomes on the basis of the mode of individual laboratory predictions.

Table 3.3: *Contingency Table for the EpiSkin test method according to the EU DSD and UN GHS classification system in the EpiSkin™. Blue: False Positive (FP), Red: False Negative (FN) predictions.*

EpiSkin test method (SIVS)

a **EU DSD**

		in vitro I	*in vitro* NI	Σ
in vivo	I	18	7	25
	NI	6	27	33
	Σ	24	34	***58***

Sensitivity (%)	72.0
Specificity (%)	81.8
Accuracy (%)	77.6

b **UN GHS**

		in vitro I	*in vitro* NI	Σ
in vivo	I	11	2	13
	NI	13	32	45
	Σ	24	34	***58***

Sensitivity (%)	84.6
Specificity (%)	71.1
Accuracy (%)	74.1

Table 3.4: *EpiSkin SIVS study. Individual results from all three testing laboratories: Means of cell viability measurements via MTT (normalised to controls) and individual laboratory predictions. Moreover the mode of the three laboratory predictions is given which serves as a basis for the categorical decision (Table 3.5) under either EU DSD or UN GHS.*

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
1	2	1-bromo-4-chlorobutane	6940-78-9	0	No	No cat.	No cat.	5.95	4.02	4.4	**1**	**1**	**1**	**1**
2	6	3-diethylaminopropionitrile	0204-5351	0	No	No cat.	No cat.	27.66	51.2	63.07	**1**	0	0	**0**
3	7	3-mercaptohexanol	51755-83-0	0	No	No cat.	No cat.	62.63	15.52	44.36	0	**1**	**1**	**1**
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	4166-20-5	0	No	No cat.	No cat.	114.34	109.48	98.18	0	0	0	**0**
5	22	diethyl phthalate	84-66-2	0	No	No cat.	No cat.	95.31	75.04	92.54	0	0	0	**0**
6	24	di-propylene glycol	25265-71-8	0	No	No cat.	No cat.	106.24	93.56	90.59	0	0	0	**0**
7	25	dipropylene glycol monobutyl ether	29911-28-2	0	No	No cat.	No cat.	105.32	93.24	103.08	0	0	0	**0**
8	26	3,4-dimethyl-1H-pyrazole	2820-37-3	0	No	No cat.	No cat.	7.02	31.3	2.6	**1**	**1**	**1**	**1**
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy] phenyl] piperazine-1-carboxylate	67914-69-6	0	No	No cat.	No cat.	118.51	116.1	113.74	0	0	0	**0**
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	7089-59-0	0	No	No cat.	No cat.	99.72	111.79	102.27	0	0	0	**0**
11	35	cyclohexadecanone	2550-52-9	0	No	No cat.	No cat.	121.85	112.38	114.87	0	0	0	**0**
12	41	naphthalene acetic acid	86-87-3	0	No	No cat.	No cat.	96.39	88.6	91.96	0	0	0	**0**
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	180898-37-7	0	No	No cat.	No cat.	100.3	93.4	95.25	0	0	0	**0**
14	48	2-(formylamino)-3-thiophenecarboxylic acid	43028-69-9	0	No	No cat.	No cat.	95.82	89.02	93.43	0	0	0	**0**
15	53	silane A-1430	2530-87-2	0	No	No cat.	No cat.	68.9	57.5	76.04	0	0	0	**0**
16	57	triethylene glycol	112-27-6	0	No	No cat.	No cat.	97.97	100.56	101.49	0	0	0	**0**
17	9	2,6-dimethyl-4-nitrobenzeneamine	16947-63-0	0.3	No	No cat.	No cat.	107.13	100.53	104.36	0	0	0	**0**
18	11	allyl phenoxyacetate	7493-74-5	0.3	No	No cat.	No cat.	96.82	96.39	102.14	0	0	0	**0**
19	36	isopropanol	67-63-0	0.3	No	No cat.	No cat.	100.41	81.58	82.39	0	0	0	**0**
20	12	2-ethylhexyl 4-aminobenzoate	26218-04-2	0.7	No	No cat.	No cat.	111.44	90.87	105.37	0	0	0	**0**
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0319002-92-1	0.7	No	No cat.	No cat.	69.37	58.06	85.07	0	0	0	**0**
22	5	3-chloro-4-fluoronitrobenzene	350-30-1	1	No	No cat.	No cat.	54.9	10.96	21.62	0	**1**	**1**	**1**
23	8	4-methylthio-benzaldehyde	3446-89-7	1	No	No cat.	No cat.	51.51	13.91	34.69	0	**1**	**1**	**1**
24	16	capryl-isostearate	209802-43-7	1	No	No cat.	No cat.	99	95.95	102.33	0	0	0	**0**
25	39	methyl stearate	112-61-8	1	No	No cat.	No cat.	103.99	90.33	101.08	0	0	0	**0**
26	44	phenylethylalcohol	60-12-8	1	No	No cat.	No cat.	92.55	83.03	95.99	0	0	0	**0**
27	30	Mixture of: diethyl cis-1,4-cyclohexanedicarboxylate; and diethyl trans-1,4-cyclohexanedicarboxylate	0072903-27-6	1.3	No	No cat.	No cat.	81.9	67.94	99.92	0	0	0	**0**

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
28	54	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl) pent-4-en-1-one (CAS 224031-70-3) and 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS 224031-71-4)	224031-70-3	1.3	No	No cat.	No cat.	66.43	40.4	55.86	0	**1**	0	**0**
29	10	allyl heptanoate	142-19-8	1.7	No	Opt Cat 3	No cat.	101.13	99.53	101.33	0	0	0	**0**
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	128119-70-0	1.7	No	Opt Cat 3	No cat.	10.81	4.44	9.73	**1**	**1**	**1**	**1**
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; and 5-endo-decylbicyclo[2.2.1]hept-2-ene	22094-85-5	1.7	No	Opt Cat 3	No cat.	103.47	107	104.22	0	0	0	**0**
32	33	heptyl butyrate	5870-93-9	1.7	No	Opt Cat 3	No cat.	103.99	102.32	111.54	0	0	0	**0**
33	50	2-phenylhexanenitrile	3508-98-3	1.7	No	Opt Cat 3	No cat.	116.18	86.67	112.93	0	0	0	**0**
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	68047-07-4	2	R38	Opt Cat 3	No cat.	4.97	4.84	13.44	**1**	**1**	**1**	**1**
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclo-penten-1'-yl)-4-penten-1-ol and 56% (1'R,2R) & 40%(1'R,2S) isomer	014864-90-6	2	R38	Opt Cat 3	No cat.	11.09	6.2	9.01	**1**	**1**	**1**	**1**
36	31	A mixture of isomers: ethyl exo-tricyclo [5.2.1.0 (2,6)] decane-endo-2-carboxylate; and ethyl endo-tricyclo [5.2.1.0(2,6)]decane-exo-2-carboxylate	80657-64-3 (mix).	2	R38	Opt Cat 3	No cat.	12.05	11.43	7.78	**1**	**1**	**1**	**1**
37	34	hexyl salicylate	6259-76-3	2	R38	Opt Cat 3	No cat.	99.85	101.87	94.56	0	0	0	**0**
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; and 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	181258-87-7 (cis), 181258-89-9 (trans)	2	R38	Opt Cat 3	No cat.	53.55	31.32	48.18	0	**1**	**1**	**1**
39	46	4-methyl-8-methylenetricyclo [3.3.1.1 (3,7)] decan-2-ol	122760-84-3	2	R38	Opt Cat 3	No cat.	12.36	7.55	4.54	**1**	**1**	**1**	**1**
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	122760-85-4	2	R38	Opt Cat 3	No cat.	14.82	31.5	13.33	**1**	**1**	**1**	**1**
41	49	isostearic acid monoisopropanolamide	152848-22-1	2	R38	Opt Cat 3	No cat.	95.69	84.85	96.59	0	0	0	**0**
42	51	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS 191044-60-7) and 1-(3-isopropylphenyl) -1-phenylethane (CAS 191044-59-4) and 1-(4-isopropylphenyl)-1-phenylethane (CAS 2320-06-1)	52783-21-8 (mix.)	2	R38	Opt Cat 3	No cat.	85.67	84.2	66.65	0	0	0	**0**
43	55	terpinyl acetate	80-26-2	2	R38	Opt	No cat.	52.96	6.26	53.77	0	**1**	0	**0**

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
44	58	tri-isobutyl phosphate	126-71-6	2	R38	Cat 3 Opt	No cat.	7.12	5.89	6.9	**1**	**1**	**1**	**1**
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	not allocated	2	R38	Cat 3 Opt	No cat.	88.36	6.34	78.81	0	**1**	0	**0**
46	4	1-decanol	112-30-1	2.3	R38	Cat 3 Cat. 2	Cat. 2	7.31	6.45	6.94	1	1	1	**1**
47	20	cyclamen aldehyde	103-95-7	2.3	R38	Cat. 2	Cat. 2	24.37	8.53	42.48	1	1	1	**1**
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	86604-75-3	2.7	R38	Cat. 2	Cat. 2	5.67	4.66	3.92	1	1	1	**1**
49	3	1-bromohexane	111-25-1	2.7	R38	Cat. 2	Cat. 2	26.22	18.88	46.79	1	1	1	**1**
50	15	a-terpineol	98-55-5	2.7	R38	Cat. 2	Cat. 2	15.16	2.75	6.4	1	1	1	**1**
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	107898-54-4	2.7	R38	Cat. 2	Cat. 2	11.71	9.79	8.83	1	1	1	**1**
52	18	butyl methacrylate	97-88-1	3	R38	Cat. 2	Cat. 2	11.25	21.13	27.93	1	1	1	**1**
53	23	di-n-propyl disulphide	629-19-6	3	R38	Cat. 2	Cat. 2	52.04	11.17	76.12	**0**	1	**0**	**0**
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	not allocated	3	R38	Cat. 2	Cat. 2	9.15	9.62	11.09	1	1	1	**1**
55	40	1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	R38	Cat. 2	Cat. 2	9.47	17.4	11.04	1	1	1	**1**
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name from company)*	7340-90-1	3.3	R38	Cat. 2	Cat. 2	12.74	87.75	14.19	1	**0**	1	**1**
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	129228-21-3	4	R38	Cat. 2	Cat. 2	81.03	21.48	89.54	**0**	1	**0**	**0**
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	125474-34-2	4	R38	Cat. 2	Cat. 2	21.91	14.76	17.12	1	1	1	**1**

Green = non-irritant categories; violet = irritant categories; Red line = in vivo threshold EU DSD and UN GHS system; 0 = in vitro classification as non-irritant; 1: in vitro classification as irritant; The final decision is calculated as the median (or mode) of the individual laboratory predictions. This decision of the in vitro test can then be translated into a categorical decision for classification and labelling (next table).

Table 3.5: *Mode [median] of the individual laboratory predictions of the EpiSkin™ SIVS study based on the EU classification system EU DSD and the UN GHS system (=table 14 of BfR). Note that the predictions do not change except for those chemicals in the narrow band between in vivo score 2.0 and 2.3. In this band the predictions (categorical decision) flips due to the decision to regard these chemicals as non-irritant (UN GHS). In this band (previously considered 'irritant') previous False Negatives (FN) become True Negatives (TN), whereas previous True Positives (TP) become False Positives. Out of the 58 substances tested this band comprises only 12 substances. Notably this band is at the cut-off (borderline) zone where False Predictions in any Test System are accumulating.*

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
1	2	1-bromo-4-chlorobutane	6940-78-9	0	No	No cat.	No	**1**	**FP**	**FP**
2	6	3-diethylaminopropionitrile	0204-5351	0	No	No cat.	No	**0**	TN	TN
3	7	3-mercaptohexanol	51755-83-0	0	No	No cat.	No	**1**	**FP**	**FP**
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	4166-20-5	0	No	No cat.	No	**0**	TN	TN
5	22	diethyl phthalate	84-66-2	0	No	No cat.	No	**0**	TN	TN
6	24	di-propylene glycol	25265-71-8	0	No	No cat.	No	**0**	TN	TN
7	25	dipropylene glycol monobutyl ether	29911-28-2	0	No	No cat.	No	**0**	TN	TN
8	26	3,4-dimethyl-1H-pyrazole	2820-37-3	0	No	No cat.	No	**1**	**FP**	**FP**
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy] phenyl] piperazine-1-carboxylate	67914-69-6	0	No	No cat.	No	**0**	TN	TN
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	7089-59-0	0	No	No cat.	No	**0**	TN	TN
11	35	cyclohexadecanone	2550-52-9	0	No	No cat.	No	**0**	TN	TN
12	41	naphthalene acetic acid	86-87-3	0	No	No cat.	No	**0**	TN	TN
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	180898-37-7	0	No	No cat.	No	**0**	TN	TN
14	48	2-(formylamino)-3-thiophenecarboxylic acid	43028-69-9	0	No	No cat.	No	**0**	TN	TN
15	53	silane A-1430	2530-87-2	0	No	No cat.	No	**0**	TN	TN
16	57	triethylene glycol	112-27-6	0	No	No cat.	No	**0**	TN	TN
17	9	2,6-dimethyl-4-nitrobenzeneamine	16947-63-0	0.3	No	No cat.	No	**0**	TN	TN
18	11	allyl phenoxyacetate	7493-74-5	0.3	No	No cat.	No	**0**	TN	TN
19	36	isopropanol	67-63-0	0.3	No	No cat.	No	**0**	TN	TN
20	12	2-ethylhexyl 4-aminobenzoate	26218-04-2	0.7	No	No cat.	No	**0**	TN	TN
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0319002-92-1	0.7	No	No cat.	No	**0**	TN	TN
22	5	3-chloro-4-fluoronitrobenzene	350-30-1	1	No	No cat.	No	**1**	**FP**	**FP**
23	8	4-methylthio-benzaldehyde	3446-89-7	1	No	No cat.	No	**1**	**FP**	**FP**
24	16	capryl-isostearate	209802-43-7	1	No	No cat.	No	**0**	TN	TN
25	39	methyl stearate	112-61-8	1	No	No cat.	No	**0**	TN	TN

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
26	44	phenylethylalcohol	60-12-8	1	No	No cat.	No	**0**	TN	TN
27	30	Mixture of: diethyl cis-1,4-cyclohexanedicarboxylate; and diethyl trans-1,4-cyclohexanedicarboxylate	0072903-27-6	1.3	No	No cat.	No	**0**	TN	TN
28	54	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl) pent-4-en-1-one (CAS 224031-70-3) and 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS 224031-71-4)	224031-70-3	1.3	No	No cat.	No	**0**	TN	TN
29	10	allyl heptanoate	142-19-8	1.7	No	Opt Cat 3	No	**0**	TN	TN
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	128119-70-0	1.7	No	Opt Cat 3	No	**1**	**FP**	**FP**
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; and 5-endo-decylbicyclo[2.2.1]hept-2-ene	22094-85-5	1.7	No	Opt Cat 3	No	**0**	TN	TN
32	33	heptyl butyrate	5870-93-9	1.7	No	Opt Cat 3	No	**0**	TN	TN
33	50	2-phenylhexanenitrile	3508-98-3	1.7	No	Opt Cat 3	No	**0**	TN	TN
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	68047-07-4	2	R38	Opt Cat 3	No	**1**	TP	**FP**
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol and 56% (1'R,2R) & 40%(1'R,2S) isomer	014864-90-6	2	R38	Opt Cat 3	No	**1**	TP	**FP**
36	31	A mixture of isomers: ethyl exo-tricyclo [5.2.1.0 (2,6)] decane-endo-2-carboxylate; and ethyl endo-tricyclo [5.2.1.0(2,6)]decane-exo-2-carboxylate	80657-64-3 (mix).	2	R38	Opt Cat 3	No	**1**	TP	**FP**
37	34	hexyl salicylate	6259-76-3	2	R38	Opt Cat 3	No	**0**	**FN**	TN
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; and 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	181258-87-7 (cis), 181258-89-9 (trans)	2	R38	Opt Cat 3	No	**1**	TP	**FP**
39	46	4-methyl-8-methylenetricyclo [3.3.1.1 (3,7)] decan-2-ol	122760-84-3	2	R38	Opt Cat 3	No	**1**	TP	**FP**
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	122760-85-4	2	R38	Opt Cat 3	No	**1**	TP	**FP**
41	49	isostearic acid monoisopropanolamide	152848-22-1	2	R38	Opt Cat 3	No	**0**	**FN**	TN
42	51	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS 191044-60-7) and 1-(3-isopropylphenyl) -1-phenylethane (CAS 191044-59-4) and 1-(4-isopropylphenyl)-1-phenylethane (CAS 2320-06-1)	52783-21-8 (mix.)	2	R38	Opt Cat 3	No	**0**	**FN**	TN
43	55	terpinyl acetate	80-26-2	2	R38	Opt Cat 3	No	**0**	**FN**	TN
44	58	tri-isobutyl phosphate	126-71-6	2	R38	Opt Cat 3	No	**1**	TP	**FP**

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	not allocated	2	R38	Opt Cat 3	No	**0**	**FN**	TN
46	4	1-decanol	112-30-1	2.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
47	20	cyclamen aldehyde	103-95-7	2.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	86604-75-3	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
49	3	1-bromohexane	111-25-1	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
50	15	a-terpineol	98-55-5	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	107898-54-4	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
52	18	butyl methacrylate	97-88-1	3	R38	Cat. 2	Cat. 2	**1**	TP	TP
53	23	di-n-propyl disulphide	629-19-6	3	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	not allocated	3	R38	Cat. 2	Cat. 2	**1**	TP	TP
55	40	1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name from company)*	7340-90-1	3.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	129228-21-3	4	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	125474-34-2	4	R38	Cat. 2	Cat. 2	**1**	TP	TP
								Specificity (%)	**81.8**	**71.1**
								Sensitivity (%)	**72**	**84.6**
								Overall Accuracy (%)	**77.6**	**74.1**

Green = non-irritant categories; violet = irritant categories; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system (dashed line indicates the shift in threshold from EU DSD to UN GHS; 0 = in vitro classification as non-irritant; 1: in vitro classification as irritant; The final decision is calculated as the median (or mode) of the individual laboratory predictions as provided in the columns EU DSD and UN GHS.

3.2.2 Performance of the original EpiDerm Test Method under EU DSD versus UN GHS system during the SIVS

This section provides all relevant data and test outcomes for the original EpiDerm test method during the SIVS. Table 3.6 provides the contingency tables for the original EpiDerm test method upon completion of the SIVS based on the EU DSD system (3.6 c) and the UN GHS system (3.6 d). Table 3.7 presents the individual test results from all laboratories for all 58 chemicals. Table 3.8 provides the test outcomes on the basis of the mode of individual laboratory predictions.

Table 3.6: *Contingency Table for the EpiDerm test method under EU DSD (c) and UN GHS (d).*

original EpiDerm test method (SIVS)

c **EU DSD**

		in vitro I	*in vitro* NI	*in vitro* Σ
in vivo	**I**	**15**	**10**	25
	NI	**4**	**29**	33
	Σ	19	39	***58***

Sensitivity (%)	**60.0**
Specificity (%)	**87.9**
Accuracy (%)	**75.9**

d **UN GHS**

		in vitro I	*in vitro* NI	*in vitro* Σ
in vivo	**I**	**8**	**5**	13
	NI	**11**	**34**	45
	Σ	19	39	***58***

Sensitivity (%)	**61.5**
Specificity (%)	**75.6**
Accuracy (%)	**72.4**

Table 3.7: *EpiDerm SIVS study. Individual results from all three testing laboratories: Means of cell viability measurements via MTT (normalised to controls) and individual laboratory predictions. Moreover the mode of the three laboratory predictions is given that serves as a basis for the categorical decision (table 3.8) under either EU DSD or UN GHS.*

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
1	2	1-bromo-4-chlorobutane	6940-78-9	0	No	No cat.	No cat.	88.85	48.22	23.2	0	**1**	**1**	**1**
2	6	3-diethylaminopropionitrile	0204-5351	0	No	No cat.	No cat.	76.8	23.41	30.33	0	**1**	**1**	**1**
3	7	3-mercaptohexanol	51755-83-0	0	No	No cat.	No cat.	51.44	43.02	63.39	0	**1**	0	**0**
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	4166-20-5	0	No	No cat.	No cat.	89.99	80.3	91.2	0	0	0	**0**
5	22	diethyl phthalate	84-66-2	0	No	No cat.	No cat.	99.22	98.9	102.15	0	0	0	**0**
6	24	di-propylene glycol	25265-71-8	0	No	No cat.	No cat.	100.35	91.79	99.67	0	0	0	**0**
7	25	dipropylene glycol monobutyl ether	29911-28-2	0	No	No cat.	No cat.	98.19	102.54	99.34	0	0	0	**0**
8	26	3,4-dimethyl-1H-pyrazole	2820-37-3	0	No	No cat.	No cat.	8.7	14.07	7.58	**1**	**1**	**1**	**1**
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy] phenyl] piperazine-1-carboxylate	67914-69-6	0	No	No cat.	No cat.	95.08	88.58	82.95	0	0	0	**0**
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	7089-59-0	0	No	No cat.	No cat.	100.19	97.66	106.13	0	0	0	**0**
11	35	cyclohexadecanone	2550-52-9	0	No	No cat.	No cat.	94.82	113.61	7.85	0	0	**1**	**0**
12	41	naphthalene acetic acid	86-87-3	0	No	No cat.	No cat.	98.32	98.65	103.69	0	0	0	**0**
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimida-zole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	180898-37-7	0	No	No cat.	No cat.	101.49	106.21	92.87	0	0	0	**0**
14	48	2-(formylamino)-3-thiophenecarboxylic acid	43028-69-9	0	No	No cat.	No cat.	100.44	97.8	105.7	0	0	0	**0**
15	53	silane A-1430	2530-87-2	0	No	No cat.	No cat.	98.62	98.55	61.72	0	0	0	**0**
16	57	triethylene glycol	112-27-6	0	No	No cat.	No cat.	94.95	94.99	95.29	0	0	0	**0**
17	9	2,6-dimethyl-4-nitrobenzeneamine	16947-63-0	0.3	No	No cat.	No cat.	97.21	107.38	101.3	0	0	0	**0**
18	11	allyl phenoxyacetate	7493-74-5	0.3	No	No cat.	No cat.	95.46	99.66	100.79	0	0	0	**0**
19	36	isopropanol	67-63-0	0.3	No	No cat.	No cat.	97.88	85.07	93.94	0	0	0	**0**
20	12	2-ethylhexyl 4-aminobenzoate	26218-04-2	0.7	No	No cat.	No cat.	104.55	91.94	107.34	0	0	0	**0**
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0319002-92-1	0.7	No	No cat.	No cat.	96.78	99.34	86.88	0	0	0	**0**
22	5	3-chloro-4-fluoronitrobenzene	350-30-1	1	No	No cat.	No cat.	101.67	53.19	84.82	0	0	0	**0**
23	8	4-methylthio-benzaldehyde	3446-89-7	1	No	No cat.	No cat.	96.12	83.85	52.76	0	0	0	**0**
24	16	capryl-isostearate	209802-43-7	1	No	No cat.	No cat.	101.06	108.02	99.46	0	0	0	**0**
25	39	methyl stearate	112-61-8	1	No	No cat.	No cat.	97.42	101.57	104	0	0	0	**0**
26	44	phenylethylalcohol	60-12-8	1	No	No cat.	No cat.	71.36	45.68	92.91	0	**1**	0	**0**
27	30	Mixture of: diethyl cis-1,4-cyclohexanedicarboxy-late; and diethyl trans-1,4-cyclohexanedicar-boxylate	0072903-27-6	1.3	No	No cat.	No cat.	97.75	100.08	106.67	0	0	0	**0**

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
28	54	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl) pent-4-en-1-one (CAS 224031-70-3) and 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS 224031-71-4)	224031-70-3	1.3	No	No cat.	No cat.	79.36	51.21	87.65	0	0	0	**0**
29	10	allyl heptanoate	142-19-8	1.7	No	Opt Cat 3	No cat.	101.07	102.09	97.01	0	0	0	**0**
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	128119-70-0	1.7	No	Opt Cat 3	No cat.	11.43	7.9	10.57	**1**	**1**	**1**	**1**
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; and 5-endo-decylbicyclo[2.2.1]hept-2-ene	22094-85-5	1.7	No	Opt Cat 3	No cat.	101.24	109.22	104.61	0	0	0	**0**
32	33	heptyl butyrate	5870-93-9	1.7	No	Opt Cat 3	No cat.	100.48	93.76	98.67	0	0	0	**0**
33	50	2-phenylhexanenitrile	3508-98-3	1.7	No	Opt Cat 3	No cat.	73.87	82.14	80.92	0	0	0	**0**
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	68047-07-4	2	R38	Opt Cat 3	No cat.	68.26	43.27	46.16	0	**1**	**1**	**1**
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclo-penten-1'-yl)-4-penten-1-ol and 56% (1'R,2R) & 40%(1'R,2S) isomer	014864-90-6	2	R38	Opt Cat 3	No cat.	11.41	8.53	14.47	**1**	**1**	**1**	**1**
36	31	A mixture of isomers: ethyl exo-tricyclo [5.2.1.0 (2,6)] decane-endo-2-carboxylate; and ethyl endo-tricyclo [5.2.1.0(2,6)]decane-exo-2-carboxylate	80657-64-3 (mix).	2	R38	Opt Cat 3	No cat.	18.27	58.78	20.39	**1**	0	**1**	**1**
37	34	hexyl salicylate	6259-76-3	2	R38	Opt Cat 3	No cat.	102.17	96.66	89.72	0	0	0	**0**
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; and 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	181258-87-7 (cis), 181258-89-9 (trans)	2	R38	Opt Cat 3	No cat.	103.72	101.71	100.12	0	0	0	**0**
39	46	4-methyl-8-methylenetricyclo [3.3.1.1 (3,7)] decan-2-ol	122760-84-3	2	R38	Opt Cat 3	No cat.	11.33	17.12	8.25	**1**	**1**	**1**	**1**
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	122760-85-4	2	R38	Opt Cat 3	No cat.	17.6	19.52	12.73	**1**	**1**	**1**	**1**
41	49	isostearic acid monoisopropanolamide	152848-22-1	2	R38	Opt Cat 3	No cat.	96.59	94.24	99.43	0	0	0	**0**
42	51	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS 191044-60-7) and 1-(3-isopropylphenyl) -1-phenylethane (CAS 191044-59-4) and 1-(4-isopropylphenyl)-1-phenylethane (CAS 2320-06-1)	52783-21-8 (mix.)	2	R38	Opt Cat 3	No cat.	107.62	116.25	116.45	0	0	0	**0**
43	55	terpinyl acetate	80-26-2	2	R38	Opt	No cat.	81.53	10.16	80.45	0	**1**	0	**0**

Count	no.	substance name	CAS-no.	Score	Classification			MTT mean of all runs			Prediction			Mode
					EU DSD	UN GHS*	UN GHS	Lab 1	Lab 2	Lab 3	Lab 1	Lab 2	Lab 3	
44	58	tri-isobutyl phosphate	126-71-6	2	R38	Cat 3 Opt	No cat.	29.97	44.63	24.33	**1**	**1**	**1**	**1**
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	not allocated	2	R38	Cat 3 Opt	No cat.	8.82	6.55	13.7	**1**	**1**	**1**	**1**
46	4	1-decanol	112-30-1	2.3	R38	Cat 3 Cat. 2	Cat. 2	12.66	7.11	58.65	1	1	**0**	**1**
47	20	cyclamen aldehyde	103-95-7	2.3	R38	Cat. 2	Cat. 2	21.6	8.67	26.39	1	1	1	**1**
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	86604-75-3	2.7	R38	Cat. 2	Cat. 2	6.39	5.61	6.43	1	1	1	**1**
49	3	1-bromohexane	111-25-1	2.7	R38	Cat. 2	Cat. 2	96.39	100.41	95.92	**0**	**0**	**0**	**0**
50	15	a-terpineol	98-55-5	2.7	R38	Cat. 2	Cat. 2	65.34	17.86	70.87	**0**	1	**0**	**0**
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	107898-54-4	2.7	R38	Cat. 2	Cat. 2	11.02	7.8	14.07	1	1	1	**1**
52	18	butyl methacrylate	97-88-1	3	R38	Cat. 2	Cat. 2	98.11	92.64	96.88	**0**	**0**	**0**	**0**
53	23	di-n-propyl disulphide	629-19-6	3	R38	Cat. 2	Cat. 2	95.68	100.78	92.09	**0**	**0**	**0**	**0**
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	not allocated	3	R38	Cat. 2	Cat. 2	42.6	47.28	31	1	1	1	**1**
55	40	1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	R38	Cat. 2	Cat. 2	21.96	55.07	39.88	1	**0**	1	**1**
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name from company)*	7340-90-1	3.3	R38	Cat. 2	Cat. 2	14.58	12.18	9.89	1	1	1	**1**
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	129228-21-3	4	R38	Cat. 2	Cat. 2	101.56	65.02	103.58	**0**	**0**	**0**	**0**
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	125474-34-2	4	R38	Cat. 2	Cat. 2	17.46	7.76	40.27	1	1	1	**1**

Table 3.8: *EpiDerm SIVS study. Individual results from all three testing laboratories: Means of cell viability measurements via MTT (normalised to controls) and individual laboratory predictions. Moreover the mode of the three laboratory predictions is given that serves as a basis for the categorical decision (next table) under either EU DSD or UN GHS.*

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
1	2	1-bromo-4-chlorobutane	6940-78-9	0	No	No cat.	No	**1**	**FP**	**FP**
2	6	3-diethylaminopropionitrile	0204-5351	0	No	No cat.	No	**1**	**FP**	**FP**
3	7	3-mercaptohexanol	51755-83-0	0	No	No cat.	No	**0**	TN	TN
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	4166-20-5	0	No	No cat.	No	**0**	TN	TN
5	22	diethyl phthalate	84-66-2	0	No	No cat.	No	**0**	TN	TN
6	24	di-propylene glycol	25265-71-8	0	No	No cat.	No	**0**	TN	TN
7	25	dipropylene glycol monobutyl ether	29911-28-2	0	No	No cat.	No	**0**	TN	TN
8	26	3,4-dimethyl-1H-pyrazole	2820-37-3	0	No	No cat.	No	**1**	**FP**	**FP**
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy] phenyl] piperazine-1-carboxylate	67914-69-6	0	No	No cat.	No	**0**	TN	TN
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	7089-59-0	0	No	No cat.	No	**0**	TN	TN
11	35	cyclohexadecanone	2550-52-9	0	No	No cat.	No	**0**	TN	TN
12	41	naphthalene acetic acid	86-87-3	0	No	No cat.	No	**0**	TN	TN
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	180898-37-7	0	No	No cat.	No	**0**	TN	TN
14	48	2-(formylamino)-3-thiophenecarboxylic acid	43028-69-9	0	No	No cat.	No	**0**	TN	TN
15	53	silane A-1430	2530-87-2	0	No	No cat.	No	**0**	TN	TN
16	57	triethylene glycol	112-27-6	0	No	No cat.	No	**0**	TN	TN
17	9	2,6-dimethyl-4-nitrobenzeneamine	16947-63-0	0.3	No	No cat.	No	**0**	TN	TN
18	11	allyl phenoxyacetate	7493-74-5	0.3	No	No cat.	No	**0**	TN	TN
19	36	isopropanol	67-63-0	0.3	No	No cat.	No	**0**	TN	TN
20	12	2-ethylhexyl 4-aminobenzoate	26218-04-2	0.7	No	No cat.	No	**0**	TN	TN
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0319002-92-1	0.7	No	No cat.	No	**0**	TN	TN
22	5	3-chloro-4-fluoronitrobenzene	350-30-1	1	No	No cat.	No	**0**	TN	TN
23	8	4-methylthio-benzaldehyde	3446-89-7	1	No	No cat.	No	**0**	TN	TN
24	16	capryl-isostearate	209802-43-7	1	No	No cat.	No	**0**	TN	TN
25	39	methyl stearate	112-61-8	1	No	No cat.	No	**0**	TN	TN
26	44	phenylethylalcohol	60-12-8	1	No	No cat.	No	**0**	TN	TN
27	30	Mixture of: diethyl cis-1,4-cyclohexanedicarboxylate; and diethyl trans-1,4-cyclohexanedicarboxylate	0072903-27-6	1.3	No	No cat.	No	**0**	TN	TN

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
28	54	Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl) pent-4-en-1-one (CAS 224031-70-3) and 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (CAS 224031-71-4)	224031-70-3	1.3	No	No cat.	No	**0**	TN	TN
29	10	allyl heptanoate	142-19-8	1.7	No	Opt Cat 3	No	**0**	TN	TN
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	128119-70-0	1.7	No	Opt Cat 3	No	**1**	**FP**	**FP**
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; and 5-endo-decylbicyclo[2.2.1]hept-2-ene	22094-85-5	1.7	No	Opt Cat 3	No	**0**	TN	TN
32	33	heptyl butyrate	5870-93-9	1.7	No	Opt Cat 3	No	**0**	TN	TN
33	50	2-phenylhexanenitrile	3508-98-3	1.7	No	Opt Cat 3	No	**0**	TN	TN
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	68047-07-4	2	R38	Opt Cat 3	No	**1**	TP	**FP**
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol and 56% (1'R,2R) & 40%(1'R,2S) isomer	014864-90-6	2	R38	Opt Cat 3	No	**1**	TP	**FP**
36	31	A mixture of isomers: ethyl exo-tricyclo [5.2.1.0 (2,6)] decane-endo-2-carboxylate; and ethyl endo-tricyclo [5.2.1.0(2,6)]decane-exo-2-carboxylate	80657-64-3 (mix).	2	R38	Opt Cat 3	No	**1**	TP	**FP**
37	34	hexyl salicylate	6259-76-3	2	R38	Opt Cat 3	No	**0**	**FN**	TN
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; and 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	181258-87-7 (cis), 181258-89-9 (trans)	2	R38	Opt Cat 3	No	**0**	**FN**	TN
39	46	4-methyl-8-methylenetricyclo [3.3.1.1 (3,7)] decan-2-ol	122760-84-3	2	R38	Opt Cat 3	No	**1**	TP	**FP**
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	122760-85-4	2	R38	Opt Cat 3	No	**1**	TP	**FP**
41	49	isostearic acid monoisopropanolamide	152848-22-1	2	R38	Opt Cat 3	No	**0**	**FN**	TN
42	51	Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane (CAS 191044-60-7) and 1-(3-isopropylphenyl) -1-phenylethane (CAS 191044-59-4) and 1-(4-isopropylphenyl)-1-phenylethane (CAS 2320-06-1)	52783-21-8 (mix.)	2	R38	Opt Cat 3	No	**0**	**FN**	TN
43	55	terpinyl acetate	80-26-2	2	R38	Opt Cat 3	No	**0**	**FN**	TN
44	58	tri-isobutyl phosphate	126-71-6	2	R38	Opt Cat 3	No	**1**	TP	**FP**
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	not allocated	2	R38	Opt Cat 3	No	**1**	TP	**FP**

Count	no.	substance name	CAS-no.	Score	Classification			Mode	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
46	4	1-decanol	112-30-1	2.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
47	20	cyclamen aldehyde	103-95-7	2.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	86604-75-3	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
49	3	1-bromohexane	111-25-1	2.7	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
50	15	a-terpineol	98-55-5	2.7	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	107898-54-4	2.7	R38	Cat. 2	Cat. 2	**1**	TP	TP
52	18	butyl methacrylate	97-88-1	3	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
53	23	di-n-propyl disulphide	629-19-6	3	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	not allocated	3	R38	Cat. 2	Cat. 2	**1**	TP	TP
55	40	1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl *(NB: CAS name from company)*	7340-90-1	3.3	R38	Cat. 2	Cat. 2	**1**	TP	TP
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	129228-21-3	4	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	125474-34-2	4	R38	Cat. 2	Cat. 2	**1**	TP	TP
							Specificity (%)		**87.9**	**75.6**
							Sensitivity (%)		**60**	**61.5**
							Overall Accuracy (%)		**75.9**	**72.4**

3.2.3 Performance of the modified EpiDerm Test Method (update validation study based on PS) under EU DSD and UN GHS system

This section provides all relevant data and test outcomes for the modified EpiDerm test method as an outcome of the update validation study performed in 2008. Table 3.9 provides the contingency tables based on the EU DSD system (3.9 e) and the UN GHS system (3.9 f). Table 3.10 presents the individual test results from all laboratories for the 20 RC chemicals. Table 3.11 provides the test outcomes on the basis of the mode of individual laboratory predictions.

Table 3.9: *Contingency table of the modified EpiDerm test method as a result of the update validation study.*

modified EpiDerm test method (PS based study)

e EU DSD

		in vitro I	*in vitro* NI	Σ
in vivo	**I**	**8**	**2**	10
	NI	**2**	**8**	10
	Σ	10	10	***20***
Sensitivity (%)		**80.0**		
Specificity (%)		**80.0**		
Accuracy (%)		**80.0**		

f UN GHS

		in vitro I	*in vitro* NI	Σ
in vivo	**I**	**6**	**1**	7
	NI	**4**	**9**	13
	Σ	10	10	***20***
Sensitivity (%)		**85.7**		
Specificity (%)		**69.2**		
Accuracy (%)		**75.0**		

Table 3.10: *Modified EpiDerm test method / update validation study based on the 20 original reference chemicals (May 2007). Shown are the individual laboratory predictions, the mode of these predictions and the final prediction under EU DSD and UN GHS. Note that the predictions 'flip' in the narrow strip between in vivo score 2.0 and 2.3 (cells shaded in grey), since substances previously regarded as irritants are now regarded 'non-irritant' (no category) under UN GHS.*

No	Chemical	*In vivo* score[§]	EU DSD[3]	UN GHS*	UN GHS	Lab1	Lab 2	Lab 3	Lab 4	Mode	EU DSD	UN GHS
1	1-bromo-4-chlorobutane	**0**	No	No cat.	No cat.	1	1	1	1	**1**	**FP**	**FP**
2	diethyl phthalate	**0**	No	No cat.	No cat.	0	0	0	0	**0**	TN	TN
3	di-propylene glycol	**0**	No	No cat.	No cat.	0	0	0	0	**0**	TN	TN
4	naphthalene acetic acid	**0**	No	No cat.	No cat.	0	0	0	0	**0**	TN	TN
5	allyl phe0xy-acetate	**0.3**	No	No cat.	No cat.	0	0	0	0	**0**	TN	TN
6	isopropa0l	**0.3**	No	No cat.	No cat.	0	1	0	0	**0**	TN	TN
7	4-methyl-thio-benzaldehyde	**1**	No	No cat.	No cat.	1	1	1	1	**1**	**FP**	**FP**
8	methyl stearate	**1**	No	No cat.	No cat.	0	0	0	0	**0**	TN	TN
9	allyl hepta0ate	**1.7**	No	Opt cat 3	No cat.	0	0	0	0	**0**	TN	TN
10	heptyl butyrate	**1.7**	No	Opt cat 3	No cat.	0	0	0	0	**0**	TN	TN
11	hexyl salicylate	**2**	R38	Opt cat 3	No cat.	0	0	0	0	**0**	**FN**	TN
12	terpinyl acetate	**2**	R38	Opt cat 3	No cat.	1	1	1	1	**1**	TP	**FP**
13	tri-isobutyl phosphate	**2**	R38	Opt cat 3	No cat.	1	1	1	1	**1**	TP	**FP**
14	1-deca0l	**2.3**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
15	cyclamen aldehyde	**2.3**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
16	1-bromohexane	**2.7**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
17	a-terpineol	**2.7**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
18	di-n-propyl disulphide	**3**	R38	Cat. 2	Cat. 2	0	0	1	0	**0**	**FN**	**FN**
19	butyl methacrylate	**3**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
20	heptanal*	**3.4**	R38	Cat. 2	Cat. 2	1	1	1	1	**1**	TP	TP
										Specificity (%)	**80**	**69.2**
										Sensitivity (%)	**80**	**85.7**
										Overall Accuracy (%)	**80**	**75**

Green = non-irritant categories; violet = irritant categories; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system; 0 = in vitro classification as non-irritant; 1: in vitro classification as irritant; The final decision is calculated as the median (or mode) of the individual laboratory predictions as provided in the columns EU DSD and UN GHS.

Table 3.11: *[Simplified version of the previous table]. Modified EpiDerm test method / update validation study based on the 20 original reference chemicals. Shown is the mode of the individual laboratory predictions leading to the final prediction decision under EU DSD and UN GHS. Note that the predictions 'flip' in the narrow strip between in vivo score 2.0 and 2.3 (cells shaded in grey), since substances previously regarded as irritants are now regarded 'non-irritant' (no category) under UN GHS.)*

No	Chemical	*In vivo* score[§]	*EU DSD*	*UN GHS**	*UN GHS*	*Mode*	EU DSD Prediction	UN GHS Prediction
1	1-bromo-4-chlorobutane	**0**	No	No cat.	No cat.	**1**	**FP**	**FP**
2	diethyl phthalate	**0**	No	No cat.	No cat.	**0**	TN	TN
3	di-propylene glycol	**0**	No	No cat.	No cat.	**0**	TN	TN
4	naphthalene acetic acid	**0**	No	No cat.	No cat.	**0**	TN	TN
5	allyl phe0xy-acetate	**0.3**	No	No cat.	No cat.	**0**	TN	TN
6	isopropa0l	**0.3**	No	No cat.	No cat.	**0**	TN	TN
7	4-methyl-thio-benzaldehyde	**1**	No	No cat.	No cat.	**1**	**FP**	**FP**
8	methyl stearate	**1**	No	No cat.	No cat.	**0**	TN	TN
9	allyl hepta0ate	**1.7**	No	Opt cat 3	No cat.	**0**	TN	TN
10	heptyl butyrate	**1.7**	No	Opt cat 3	No cat.	**0**	TN	TN
11	hexyl salicylate	**2**	R38	Opt cat 3	No cat.	**0**	**FN**	TN
12	terpinyl acetate	**2**	R38	Opt cat 3	No cat.	**1**	TP	**FP**
13	tri-isobutyl phosphate	**2**	R38	Opt cat 3	No cat.	**1**	TP	**FP**
14	1-deca0l	**2.3**	R38	Cat. 2	Cat. 2	**1**	TP	TP
15	cyclamen aldehyde	**2.3**	R38	Cat. 2	Cat. 2	**1**	TP	TP
16	1-bromohexane	**2.7**	R38	Cat. 2	Cat. 2	**1**	TP	TP
17	a-terpineol	**2.7**	R38	Cat. 2	Cat. 2	**1**	TP	TP
18	di-n-propyl disulphide	**3**	R38	Cat. 2	Cat. 2	**0**	**FN**	**FN**
19	butyl methacrylate	**3**	R38	Cat. 2	Cat. 2	**1**	TP	TP
20	heptanal*	**3.4**	R38	Cat. 2	Cat. 2	**1**	TP	TP
						Specificity (%)	**80**	**69.2**
						Sensitivity (%)	**80**	**85.7**
						Overall Accuracy (%)	**80**	**75**

Green = non-irritant categories; violet = irritant categories; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system; 0 = in vitro classification as non-irritant; 1: in vitro classification as irritant; The final decision is calculated as the median (or mode) of the individual laboratory predictions as provided in the columns EU DSD and UN GHS.

3.2.4 Performance of the SkinEthic Test Method (catch-up validation study) under EU versus UN GHS system on the basis of 20 PS reference chemicals

This section provides all relevant data and test outcomes for the SkinEthic test method as an outcome of the catch-up study performed in 2008. Table 3.12 provides the contingency tables for the SkinEthic test method upon completion of the catch-up study based on the EU DSD system (3.12 g) and the UN GHS system (3.12 h). Table 3.13 provides the test outcomes on the basis of the mode of individual laboratory predictions for the 20 RC tested.

Table 3.12 *Contingency table of the SkinEthic test method as a result of the catch-up validation study from 2008.*

SkinEthic test method (PS based study)

g **EU DSD**

		in vitro I	*in vitro* NI	Σ
in vivo	I	**9**	1	10
	NI	**2**	**8**	10
	Σ	11	9	***20***
Sensitivity (%)		**90.0**		
Specificity (%)		**80.0**		
Accuracy (%)		**85.0**		

h **UN GHS**

		in vitro I	*in vitro* NI	Σ
in vivo	I	**7**	**0**	7
	NI	**4**	**9**	13
	Σ	11	9	***20***
Sensitivity (%)		**100.0**		
Specificity (%)		**69.2**		
Accuracy (%)		**80.0**		

Table 3.13: *SkinEthic test method / catch-up validation study based on the 20 original reference chemicals (May 2007). Shown are the Individual laboratory predictions and the mode thereof leading to the final decision under EU DSD and UN GHS. Note that the predictions 'flip' in the narrow strip between in vivo score 2.0 and 2.3 (cells shaded in grey), since substances previously regarded as irritants are now regarded 'non-irritant' (no category) under UN GHS.*

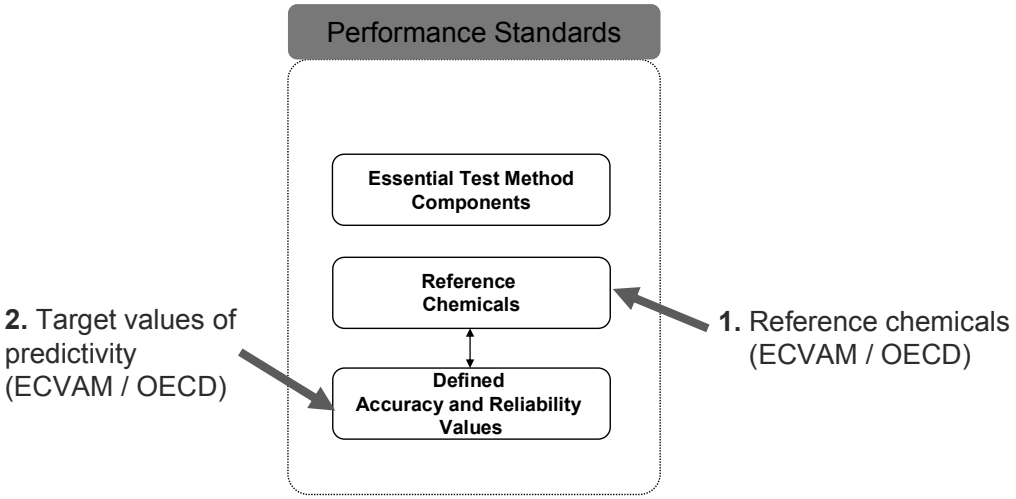
No	Chemical	*In vivo* score[§]	EU DSD	*UN GHS**	*UN GHS*	Lab A	Lab B	Lab C	Mode	Prediction EU DSD	Prediction UN GHS
1	1-bromo-4-chlorobutane	**0**	No	No cat.	No cat.	1	1	1	**1**	**FP**	**FP**
2	diethyl phthalate	**0**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
3	di-propylene glycol	**0**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
4	naphthalene acetic acid	**0**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
5	allyl phe0xy-acetate	**0.3**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
6	isopropa0l	**0.3**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
7	4-methyl-thio-benzaldehyde	**1**	No	No cat.	No cat.	1	1	1	**1**	**FP**	**FP**
8	methyl stearate	**1**	No	No cat.	No cat.	0	0	0	**0**	TN	TN
9	allyl hepta0ate	**1.7**	No	Opt cat 3	No cat.	0	0	0	**0**	TN	TN
10	heptyl butyrate	**1.7**	No	Opt cat 3	No cat.	0	0	0	**0**	TN	TN
11	hexyl salicylate	**2**	R38	Opt cat 3	No cat.	0	0	0	**0**	**FN**	TN
12	terpinyl acetate	**2**	R38	Opt cat 3	No cat.	1	1	1	**1**	TP	**FP**
13	tri-isobutyl phosphate	**2**	R38	Opt cat 3	No cat.	1	1	1	**1**	TP	**FP**
14	1-deca0l	**2.3**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
15	cyclamen aldehyde	**2.3**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
16	1-bromohexane	**2.7**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
17	a-terpineol	**2.7**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
18	di-n-propyl disulphide	**3**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
19	butyl methacrylate	**3**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
20	heptanal*	**3.4**	R38	Cat. 2	Cat. 2	1	1	1	**1**	TP	TP
								Specificity (%)		**80**	**69.2**
								Sensitivity (%)		**90**	**100**
								Overall Accuracy (%)		**85**	**80**

Green = non-irritant categories; violet = irritant categories; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system; 0 = in vitro classification as non-irritant; 1: in vitro classification as irritant; The final decision is calculated as the median (or mode) of the individual laboratory predictions as provided in the columns EU DSD and UN GHS.

4. ADAPTATION OF THE KEY ELEMENTS OF THE PERFORMANCE STANDARDS

This section provides an overview over the scientific background of the recent adaptations of the ECVAM Performance Standards (PS) for applying human skin models to *in vitro* skin irritation testing (1). These adaptations are not limited to the ECVAM PS document, but also apply to the PS as outlined in the annexes of both, the EU Test Method B.46 and the draft OECD Test Guideline on *in vitro* skin irritation testing. For an overview of the changes required in the different PS elements see Figure 4.1.

Figure 4.1: *Schematic outline of the necessary changes with regard to the three key elements of Performance Standards. Note that the changes in the context of the regulatory requirements are restricted to elements 2 and 3 of the PS, i.e. the Defined Accuracy and Reliability Values and the Reference Chemicals, respectively*



4.1 Background

4.1.1 Reason for the adaptation of the in vitro skin irritation Performance Standards

The original PS were defined in May 2007 (Annexe 1 doc09) after completion of the ECVAM Skin Irritation Validation Study (SIVS) conducted between December 2003 to August 2006 (Annexe 1 doc01-08; doc 26) and the ESAC peer review process finalised with the issuing of the ESAC statement on the reference methods in 2007 (Annexe 2). During the SIVS, the reliability, relevance and limitations (including chemical applicability domain) of two commercially available Reconstructed human Epidermis (RhE) models (EpiSkin^{TM} and EpiDerm^{TM}) were analysed. The SIVS was designed and conducted prior to the adoption of the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2008). Consequently, the SIVS evaluated the test methods under scrutiny primarily with respect to the EU classification system as described in the Dangerous Substances Directive (the "EU DSD" system) (EC 2001) albeit considering the GHS classification system during

selection of substances to be tested in the SIVS (see section 2). Thus, the original PS, including both the list of *Reference Chemicals* and the *Accuracy Target Values,* were based on the EU DSD (EC 2001), which consists of two categories: *no label* (non-classified substances) and *R38* (irritant substances) with a cut-off *in vivo* score of 2.0.

In December 2008 the EU adopted the UN GHS (United Nations 2008) and implements this by means of the Classification, Labelling and Packaging (CLP) Regulation (EC 2008). This regulation came into force on 20 January 2009 and will replace, after a transitional period, the previous EU legislations (EC 2001) for the classification of substances and mixtures (i.e. preparations). The EU classification system based on GHS (the "CLP" system) (EC 2008) directly transposes the UN GHS system (United Nations 2008) which foresees one irritant category. The EU will not use an additional optional category for mild irritants ("Category 3") that will apply only to some authorities (e.g. pesticides) (UN GHS*). Therefore the CLP system continues to use two categories to distinguish *non-classified* (No Category) from irritant (Category 2) substances. However, according to the new rules for skin irritation classification and labelling (C&L) (United Nations 2008; EC 2008), the cut-off score to distinguish between No Category and Category 2 substances was shifted to 2.3 (UN GHS or CLP) from a value of 2.0 (EU DSD). Consequently substances with an *in vivo* score between 2.0 and 2.3 that were considered irritant under the EU DSD are now non-classified under UN GHS, which does not use the optional Category 3 ($1.7 \leq$ Cat 3 < 2.3).

This had practical consequences on the ECVAM PS:
(a) the set of Reference Chemicals (RC) was not balanced any more (three former R38 substances had become not classified under UN GHS) (Griesinger et al. 2008) and although this can be regarded as reflecting the real prevalence of irritants much better, it is good practice to have a balanced distribution of RC enabling assessment of both classified (irritant) and non-classified substances on the basis of equal numbers of test substances;
(b) the accuracy target values did not match the changed prevalence which results from the cut-off shift (Griesinger et al. 2008): with a higher cut-off, more substances will not be classified in the future and, inversely, the prevalence of skin irritant substances will decrease.

Therefore, the global adoption of GHS (in the EU through regulation EC 1272/2008 - CLP regulation) (EC 2008) made necessary an update of the original ECVAM PS (Annexe 1 doc 09) in order to balance the set of RC and carefully adjust the accuracy target values (Griesinger et al. 2008). Minor adaptations include more precise specifications concerning:

1) Recommendations regarding the training set for developing similar or modified test methods that may qualify for PS-based equivalence validation studies, in particular limitations regarding the use of RC for test development/optimisation purposes.
2) The number of times that invalid runs may be retested.
3) The number of invalid run sequences (i.e. absence of 3 valid independent runs in a single laboratory) after retesting that are acceptable for the data set to be considered qualified for the purpose of an equivalence validation study.
4) The calculation of Reliability (Reproducibility) and Predictive Capacity (Accuracy)

This section of the Background Document intends to explain the rationale and details of the amendment of the PS and provide an overview on the performance (Reliability and Predictive Capacity) of the Validated Reference Method (EpiSkin^{TM}) under the UN GHS/CLP system for skin irritation C&L (United Nations, 2008; EC 2008) calculated with the new rules set out in the revised PS. These values form the basis for setting the target values for reproducibility and accuracy.

The performances of the three ECVAM-validated full-replacement *in vitro* skin irritation test methods (the EpiSkin^{TM}, the EpiDerm^{TM} SIT and the SkinEthic^{TM} RHE test methods) (ESAC 2007 and 2008, Annex 2 and 3) under the UN GHS/CLP system for skin irritation C&L (United Nations, 2008; EC 2008; ESAC

2008 Annex 4), as previously calculated, are summarised and explained in section 0 (Executive Summary) and documented in detail section 3 of this document.

4.1.2 Key elements of the Performance Standards

The evaluation and acceptance criteria or PS are structured in three main areas that are referred to as PS elements:

Element 1. Essential Test Method Components:
These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed, mechanistically and functionally similar test method. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components will help to assure that a proposed test method is based on the same concepts as the corresponding validated test method.

Element 2. List of Reference Chemicals:
These are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method. These chemicals are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method. These Reference Chemicals (RC) are the minimum that should be used to evaluate the performance of a proposed, mechanistically and functionally similar test method. If any of the recommended chemicals is unavailable, other chemicals for which adequate reference data are available could be used instead. To the extent possible, the substitute chemical(s) should be of the same chemical class and activity as the original chemical(s). If desired, additional chemicals representing other chemical or product classes and for which adequate reference data are available can be used to more comprehensively evaluate the proposed test method. However, these additional chemicals should not include any that have been used to develop the proposed test method.

Element 3. Target Values for Reliability and Predictive Capacity (Accuracy):
These are the performance requisites that should be achieved by the proposed test method when evaluated using the minimum list of RC, i.e. reliability and predictive capacity that should be achieved by the proposed test method when testing the RC.

4.2 Description of Necessary Changes

4.2.1 Reference Chemicals (Element 2 of the Performance Standards)

a) Description of changes

This change concerns element 2 of the PS, the defined set of Reference Chemicals to be used for the evaluation of similar or modified test methods (Figure 4.1). The threshold shift for classifying skin irritants discussed in section 1.1 has the consequence that 3 of the Reference Chemicals of the current PS are no longer classified as skin irritants (Cat 2). These are the substances 11 to 13 (Table 4.1).

To achieve again a balanced set of non-irritants (No Cat) and irritants (Cat 2) (taking the GHS threshold into consideration), it was necessary to remove three of the non-irritant substances and add three additional irritant substances with an in vivo score ≥ 2.3. Care was taken to **preserve the overall Predictive Capacity achieved by the Validated Reference Method (VRM), i.e. the EpiSkin test method, with the 58 chemicals** under GHS (e.g. comparative FN and FP rates) – see below (Section 4.2.2) – and to **maintain a wide range of chemical functionalities among the RC**.

Moreover, one substance in the original list of Reference Chemicals cannot be used in Japan, and for four of the irritant substances (GHS Cat. 2 based on the Draize Skin Irritation Test) in this list there is good evidence from Human 4 h Patch Tests that they are non-irritant to humans (Table 4.1). Since RhE models are based on cells of human origin, it would be contra productive to include all of these substances in the final revised list of Reference Chemicals and request new RhE test methods to wrongly predict human responses. Thus, an attempt was made to substitute them.

The proposed Reference Chemicals in the revised PS (Table 4.2) are still a representative subset of the 58 SIVS substances with regard to chemical functionalities. Furthermore, all the proposed Reference Chemicals in the revised PS were tested in all three validated full replacement test methods (EpiSkin™, modified EpiDerm™ and SkinEthic™ RHE). Some of the newly proposed Reference Chemicals were tested not in three but only in one or two laboratories (Table 4.3). However, all of these substances were tested by all of the test methods and all predictions produced for these substances showed 100% concordance between replicate tissues, between runs and between the three test methods (Table 4.3).

Importantly, all three test methods showed acceptable reproducibility both within (> 90%) and between (> 85%) laboratories during their validation, and therefore the data from a single laboratory should be sufficient to evaluate their performance regarding these Reference Chemicals.

Table 4.1 shows the new threshold value and indicates in pale yellow those non-irritant substances that have been taken out of the Reference Chemicals set (in vivo score < 2.3) to achieve a balanced set under GHS. The substance that was removed for not being usable in Japan is highlighted in pale blue. The substances know to be non-irritant to humans are depicted in bold face and marked with an asterisk, while the substances removed are highlighted in pale orange.

Table 4.1. *Original Reference Chemicals of the Performance Standards. Substances with an in vivo score < 2.3 (=no category under UN GHS) that have been deleted in the new RC are highlighted in pale yellow. Column 1: Number of RC; green: Reference Chemicals that should be considered non irritant, pink: Reference Chemicals that should be considered irritant. Column 2: chemical name of the RC. Column 3: in vivo score. Column 4: EU DSD category based on the in vivo score. Column 5: UN GHS category based on the in vivo score. Column 6: In vitro classification based on the Validated Reference Method (EpiSkin). Blue/red I: False Positives (FP), Green/red NI: False Negatives (FN). RC: Reference Chemicals. Red line: new categorical threshold under UN GHS.*

Nr.	Chemical	*In vivo* Score	EU DSD *in vivo* category	UN GHS *in vivo* category	EpiSkin classifi-cation
1	1-bromo-4-chlorobutane	0	no label	no category	**I**
2	diethyl phthalate	0	no label	no category	NI
3	di-propylene glycol	0	no label	no category	NI
4	naphthalene acetic acid	0	no label	no category	NI
5	allyl phenoxy-acetate	0.3	no label	no category	NI
6	isopropanol	0.3	no label	no category	NI
7	4-methyl-thio-benzaldehyde	1	no label	no category	**I**
8	methyl stearate	1	no label	no category	NI
9	allyl heptanoate	1.7	no label	optional cat. 3	NI
10	heptyl butyrate	1.7	no label	optional cat. 3	NI
11	hexyl salicylate	2	R38	optional cat. 3	**NI**
12	terpinyl acetate	2	R38	optional cat. 3	**NI**
13	tri-isobutyl phosphate	2	R38	optional cat. 3	I
14	**1-decanol***	2.3	R38	category 2	I
15	cyclamen aldehyde	2.3	R38	category 2	I
16	1-bromohexane	2.7	R38	category 2	I
17	**α-terpineol***	2.7	R38	category 2	I
18	**di-n-propyl disulphide***	3	R38	category 2	**NI**
19	**butyl methacrylate***	3	R38	category 2	I
20	heptanal	3.4	R38	category 2	I

Table 4.2 provides the new Reference Chemicals, with the three irritant substances (in vivo score > 2.3) included to achieve a balanced set under GHS highlighted in pale yellow, the substance introduced to replace the one problematic in Japan highlighted in pale blue and the substances that replaced the GHS Cat 2 substances known to be non-irritant in humans highlighted in pale orange. Substances known to be non-irritant to humans that still remained in the new list of Reference Chemicals are depicted in bold face and marked with an asterisk

T***able 4.2.*** *New RC of the PS. The three substances with an in vivo score > 2.3 (=irritants under GHS) that have been inserted in the new RC to arrive at balanced set of 10 non-irritants and 10 irritants are highlighted in pale orange. Column 1: Number of RC; green: Reference Chemicals that should be considered non irritant, pink: Reference Chemicals that should be considered irritant. Column 2: chemical name of the RC. Column 3: In vivo score. Column 4: EU DSD category based on the in vivo score. Column 5: UN GHS category based on the in vivo score. Column 6: In vitro category based on the Validated Reference Method (EpiSkin). Blue/red I: False Positives (FP), Green/red NI: False Negatives (FN). RC: Reference Chemicals.*

Nr.	Chemical	*In vivo* Score	EU DSD *in vivo* category	UN GHS *in vivo* category	EpiSkin classifi-cation
1	1-bromo-4-chlorobutane	0	no label	no category	I
2	diethyl phthalate	0	no label	no category	NI
3	naphthalene acetic acid	0	no label	no category	NI
4	allyl phenoxy-acetate	0.3	no label	no category	NI
5	isopropanol	0.3	no label	no category	NI
6	4-methyl-thio-benzaldehyde	1	no label	no category	I
7	methyl stearate	1	no label	no category	NI
8	heptyl butyrate	1.7	no label	optional cat. 3	NI
9	hexyl salicylate	2	R38	optional cat. 3	NI
10	cinnamaldehyde	2	R38	optional cat. 3	I
11	**1-decanol***	2.3	R38	category 2	I
12	cyclamen aldehyde	2.3	R38	category 2	I
13	1-bromohexane	2.7	R38	category 2	I
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	2.7	R38	category 2	I
15	potassium hydroxide (5% aq.)	3	R38	category 2	I
16	**di-n-propyl disulphide***	3	R38	category 2	NI
17	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	R38	category 2	I
18	1-methyl-3-phenyl-1-piperazine	3.3	R38	category 2	I
19	heptanal	3.4	R38	category 2	I
20	tetrachloroethylene	4	R38	category 2	I

In summary, the **deleted** Substances are:

1) to achieve a balanced set

- di-propylene glycol (score: 0, NI acc to GHS)
- allyl heptanoate (score:1.7, NI acc to GHS)
- terpinyl acetate (score: 2.0, NI acc to GHS)

2) substance problematic in Japan

- tri-isobutyl phosphate (score: 2.0, NI acc to GHS)

3) substances known to be non-irritant to humans

- α-terpineol (score: 2.7, I acc to GHS)
- butyl methacrylate (score: 3, I acc to GHS)

In summary, the **added** Substances are:

1) to achieve a balanced set

- 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl (score: 2.7, I acc to GHS)
- benzenethiol, 5-(1,1-dimethylethyl)-2-methyl (score: 3.3, I acc to GHS)
- methyl-3-phenyl-1-piperazine (score: 3.3, I acc to GHS)

2) to replace substance problematic in Japan

- cinnamaldehyde (score: 2.0, NI acc to GHS)

3) to replace substances known to be non-irritant to humans

- potassium hydroxide (5% aq.) (score: 3.0, I acc to GHS)
- tetrachloroethylene (score: 4.0, I acc to GHS)

b) Rationale for the deletion and insertion of substances

Replacing excess non-irritants to achieve a balanced set under UN GHS:

The aim was to delete substances covering the spectrum of irritancy scores from 0 to 2 as complete as possible. Scores of deleted chemicals were: 0, 1.7 and 2.

Specifically:

di-propylene glycol (score 0) is an intermediate and was therefore deleted in view of its, perhaps, limited availability. The bordering RC 2 & 4, which are also correctly identified by the VRM, would have also been an option for deletion but, as opposing to RC 3, are not intermediates. Moreover RC 2 (diethyl phtalate) has been proven useful for variability assessment [ECVAM in-house laboratory evaluation of a skin irritation method based on reconstructed human epidermis]

allyl heptanoate (score 1.7) is structurally very similar to heptyl butyrate (RC 10) which would have been the alternative option. Allyl heptanoate was an arbitrary choice considering these two possible options.

terpinyl acetate (score 2.0): Regarding the *in vivo* score of 2.0, in principle three options were available:
tri-isobutyl phosphate, *terpinyl acetate* and *hexyl salicylate*. Tri-isobutyl phosphate, a FP under GHS, had to be retained for reasons of preserving the overall predictivity, i.e. maintaining the number of falsely classified substances amongst the non-irritants (='false positives') within the new set of RC (this substance was however replaced by another FP substance, see below). To approximate the predictive values calculated on the basis of individual laboratory predictions of the RCs as far as possible to those gained for the 58 test substances of the SIVS, terpinyl acetate was deleted since the values obtained for this substance deviated slightly more from the overall SIVS values than those obtained for hexyl salicilate. Therefore terpinyl acetate was deleted although, as a fragrance, it would have been more relevant in this context with regard to substance class and expected exposure scenario (possible topical application) than hexyl salicylate which is a food additive.

The three new irritant substances, i.e. ***2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl*** (score: 2.7), ***benzenethiol, 5-(1,1-dimethylethyl)-2-methyl*** (score: 3.3), and ***1-methyl-3-phenyl-1-piperazine*** (score: 3.3), added in replacement of di-propylene glycol, allyl heptanoate and terpinyl acetate, were selected from

the SIVS chemicals set because they are commercially available and to increase the balance of *in vivo* irritancy scores between 2.3 and 4. Thus, two substances with an *in vivo* irritancy score of 3.3 were added since this score was not represented by any substance in the original list of RC (Tables 4.1 and 4.2).

Replacing the substance that cannot be used in Japan:

Tri-isobutyl phosphate is not available in Japan and thus cannot be used in equivalence validation studies of RhE test methods developed in Japan. Therefore, JaCVAM requested the replacement of this substance in the list of RC. Since tri-isobutyl phosphate is a false positive in the VRM under the GHS system, it needed to be replaced by another false positive substance of the VRM that is commercially available. Since no such substance could be retrieved from the 58 SIVS chemicals, a substance tested during the post pre-validation optimization phase that gave concordant results in EpiSkin™ (VRM), modified EpiDerm™ and SkinEthic™ RHE, and with the same *in vivo* irritancy score as tri-isobutyl phosphate was selected instead. The selected substance was **cinnamaldehyde** (score: 2.0).

Replacing substances known to be non-irritant to humans:

α-terpineol is a UN GHS Cat 2 substance (based on the Draize Skin Irritation Test; score: 2.7) that is known to be non-irritant to humans based on the Human 4 h Patch Test (0/59 positive reactions) (Basketter et al. 2004; Jirova et al. 2007). For this reason and as explained above, this substance was deleted. Since there were no more commercially available GHS Cat 2 substances correctly predicted by the VRM in the list of 58 SIVS chemicals, a substance tested during the post pre-validation optimization phase that gave concordant results in EpiSkin™ (VRM), modified EpiDerm™ and SkinEthic™ RHE was selected instead. The selected substance was ***tetrachloroethylene*** (score: 4.0).

Butyl methacrylate is a UN GHS Cat 2 substance (based on the Draize Skin Irritation Test; score: 3.0) that is known to be non-irritant to humans based on the Human 4 h Patch Test (0/30 positive reactions) (Jirova et al. 2007). For this reason and as explained above, this substance was deleted. Since there were no more commercially available GHS Cat 2 substances correctly predicted by the VRM in the list of 58 SIVS chemicals, a substance tested during the post pre-validation optimization phase that gave concordant results in EpiSkin™ (VRM), modified EpiDerm™ and SkinEthic™ RHE was selected instead. The selected substance was ***potassium hydroxide (5% aq.)*** (score: 3.0).

Two further substances in the original list of RC are known to be non-irritant to humans based on the Human 4 h Patch Test. These are 1-decanol (a GHS borderline chemical: score: 2.3) with 25/189 positive reactions, and di-n-propyl disulphide (a possible false negative of the VRM) with 6/30 positive reactions (Basketter et al. 2004; Jirova et al. 2007). However, none of these substances could be replaced because none the remaining commercially available GHS Cat 2 substances that were part of the list of substances tested during the post pre-validation optimization phase and which gave concordant results between the EpiSkin™ and the modified EpiDerm™ test methods was a usable option:

- 1,1,1-trichloroethane (score: 4.0; correctly identified as irritant by EpiSkin™, modified EpiDerm™ and SkinEthic™ RHE) is similar to tetrachloroethylene (same chemical class), which was already selected as a RC, and is an ozone depleting substance included in the Montreal Protocol
- 1-bromopentane (score: 2.7; correctly identified as irritant by EpiSkin™, modified EpiDerm™ and SkinEthic™ RHE) is similar to 1-bromohexane (same chemical class), which was already included in the list of RC
- lilestralis/lilial (identified as irritant by EpiSkin™, modified EpiDerm™ and SkinEthic™ RHE) does not have consistent *in vivo* data between independent experiments, i.e. two independent studies using the Draize Skin Irritation Test gave in vivo irritancy scores of 2.0 (GHS No Cat) and 2.7 (GHS Cat 2)

- methyl palmitate (score: 3.0; False Negative in EpiSkin™, modified EpiDerm™ and SkinEthic™ RHE) is also known to be non-irritant to humans based on the Human 4 h Patch Test (1/29 positive reactions) (Basketter et al. 2004)

Thus, there are no more substances that are commercially available within all of the 58 SIVS substances plus the substances tested during the post pre-validation optimization phase that could be used to further replace any substances in the list of Reference Chemicals.

The final list of 10 GHS Cat 2 RC contains substances for each of the *in vivo* irritancy scores 2.3, 2.7, 3.0, 3.3, 3.4 and 4.0, and is consequently more balanced than the original list (Tables 4.1 and 4.2).

Finally, It is important to note that heptanal, a substance also tested during the post pre-validation optimization phase but not during SIVS, was already introduced in the original list of Reference Chemicals (RC) in order to have a substance with an *in vivo* irritancy score higher than 3.3 in the list of RC (in the list of 58 SIVS there were no commercially available substances with *in vivo* irritancy score higher than 3.3 included. The two score 4 substances tested during SIVS are not commercially available).

Table 4.3 *(next page) Cell viabilities and predictions for the 6 new RC that were mostly tested in only one laboratory. Heptanal, although part of the original RC, at the time of drafting the original RC had only been tested in one laboratory with the VRM (EpiSkinTM). Note, that all substances were tested in all three test methods and gave the same prediction (True Positive, TP, highlighted in green, False Positive, FP, red) and hence were 100% concordant between the test methods and, in most cases, also with respect to the in vivo reference method. The data obtained with EpiSkin and the original EpiDerm in SIVS for substances 5, 6 and 7 are included in sections 3.2.1 and 3.2.2.*

1) Cinnamaldehyde

	EpiSkin (2005)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (2005)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1	6,7	1,0	*FP*	0,8	0,2	*FP*	5,3	1,0	*FP*	11,3	0,8	*FP*
Run 2	7,4	2,1	*FP*	1,2	0,3	*FP*	4,5	0,6	*FP*	12,4	1,0	*FP*
Run 3	6,3	0,2	*FP*	1,3	0,0	*FP*	4,1	0,4	*FP*	10,5	0,4	*FP*
Mean/SD	***6,8***	***0,56***	*FP*	***1,1***	***0,28***	*FP*	***4,7***	***0,62***	*FP*	***11,4***	***0,95***	*FP*

2) Potassium hydroxide (5% aq.)

	EpiSkin (2005)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (2005)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1	8,0	0,2	*TP*	34,6	3,3	*TP*	4,3	0,5	*TP*	11,5	1,0	*TP*
Run 2	41,4	2,8	*TP*	0,7	0,1	*TP*	3,3	0,4	*TP*	9,9	0,3	*TP*
Run 3	17,4	7,9	*TP*	14,8	2,6	*TP*	5,4	2,2	*TP*	10,2	0,4	*TP*
Mean/SD	***22,3***	***17,22***	*TP*	***16,7***	***17,05***	*TP*	***4,3***	***1,02***	*TP*	***10,5***	***0,85***	*TP*

3) Heptanal

	EpiSkin (2005)			SkinEthic RHE (mean of 3 runs in 3 Labs)			modified EpiDerm (mean of 3 runs in 5 Labs)			o' EpiDerm (2005)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run/Lab 1	9,2	0,6	*TP*	1,1	0,0	*TP*	6,4	1,7	*TP*	12,8	1,6	*TP*
Run/Lab 2	8,0	4,8	*TP*	1,5	0,1	*TP*	4,5	0,6	*TP*	9,1	0,5	*TP*
Run/Lab 3	6,4	1,0	*TP*	1,3	0,2	*TP*	5,2	0,9	*TP*	10,4	0,2	*TP*
Lab 4							5,2	1,6	*TP*			
Lab 5							5,3	0,4	*TP*			
Mean/SD	***7,9***	***1,40***	*TP*	***1,3***	***0,20***	*TP*	***5,3***	***0,65***	*TP*	***10,8***	***1,88***	*TP*

4) Tetrachloroethylene

	EpiSkin (2005)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (2005)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1	9,1	1,1	*TP*	1,1	0,3	*TP*	5,6	0,3	*TP*	11,3	0,7	*TP*
Run 2	8,0	3,5	*TP*	1,4	0,3	*TP*	4,6	0,7	*TP*	15,1	5,4	*TP*
Run 3	15,3	14,9	*TP*	1,5	0,5	*TP*	4,2	0,4	*TP*	15,7	0,6	*TP*
Mean/SD	***10,8***	***3,91***	*TP*	***1,3***	***0,21***	*TP*	***4,8***	***0,69***	*TP*	***14,0***	***2,39***	*TP*

5) Benzenethiol,5-(1,1-dimethylethyl)-2-methyl

	EpiSkin (SIVS)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (SIVS)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1				0,0	0,0	*TP*	9,9	1,5	*TP*			
Run 2				8,0	1,0	*TP*	8,4	0,6	*TP*			
Run 3				0,2	0,4	*TP*	11,5	3,3	*TP*			
Mean/SD				***2,7***	***4,56***	*TP*	***9,9***	***1,56***	*TP*			

6) 1-methyl-3-phenyl-1-piperazine

	EpiSkin (SIVS)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (SIVS)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1				8,9	2,5	*TP*	6,1	0,2	*TP*			
Run 2				14,2	2,9	*TP*	10,5	0,7	*TP*			
Run 3				1,4	0,6	*TP*	5,5	0,1	*TP*			
Mean/SD				***8,2***	***6,41***	*TP*	***7,3***	***2,73***	*TP*			

7) 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl

	EpiSkin (SIVS)			SkinEthic RHE			modified EpiDerm			o' EpiDerm (SIVS)		
	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.	Viability(%)	SD	Pred.
Run 1				0,8	0,0	*TP*	5,6	0,2	*TP*			
Run 2				0,7	0,1	*TP*	9,3	0,6	*TP*			
Run 3				0,7	0,0	*TP*	5,1	0,2	*TP*			
Mean/SD				***0,7***	***0,06***	*TP*	***6,7***	***2,29***	*TP*			

4.2.2 Defined Accuracy Values (Element 3 of the Performance Standards)

4.2.2.1 Recalculation of the Predictive Capacity of the Validated Reference Method (EpiSkin™) according to GHS

a. Background to the recalculation

The target values regarding Predictive Capacity specified in the Performance Standards were derived from the predictions obtained with the Validated Reference Method (EpiSkin™). More specifically, the values specified in the original ECVAM PS were derived from the median of the individual laboratory predictions for the 20 Reference Chemicals, using all runs and considering the EU DSD classification system. These values were 70% (sensitivity) and 80% (specificity).

However, in order to reflect the threshold change introduced by the UN GHS classification system (*in vivo* score cut-off shifted from 2.0 to 2.3) in the target values set in the PS, the overall Predictive Capacity of the VRM had to be recalculated. This was done on the basis of all 58 chemicals of the SIVS study, in order to get a picture as complete as possible reg. the consequences of the threshold change on the Predictive Capacity of the test method. The calculations were performed for both the EU DSD and UN GHS classification systems on the basis of a set of rules defined in the revised PS, which ensure that the values describing reliability and relevance are calculated in a predefined and hence consistent manner. These rules, connected by a logical AND operator, are as follows:

Rule 1. Only the data of runs from COMPLETE RUN SEQUENCES qualify for the calculation of the test method within laboratory variability (WLV), between laboratory variability (BLV), and predictive capacity (accuracy).
AND

Rule 2. The final classification for each RC in each participating laboratory should be obtained by using the MEAN VALUE OF VIABILITY over the different runs of a complete run sequence.
AND

Rule 3. Only the data obtained for chemicals that have COMPLETE RUN SEQUENCES IN ALL PARTICIPATING LABORATORIES qualify for the calculation of the test method BLV.
AND

Rule 4. The calculation of the accuracy values should be done on the basis of the INDIVIDUAL LABORATORY PREDICTIONS obtained for the 20 RC by the different participating laboratories.

In this context, a **run sequence** consists of three independent runs from one laboratory concerning one test substance and a **complete run sequence** is a run sequence where all three runs are valid. This means that any single invalid run invalidates an entire run sequence of three runs. For further explanations on the calculation method please refer to Textbox 4.1.

Table 4.4 summarises the accuracy values (specificity, sensitivity and overall accuracy) of the VRM (EpiSkin™) for the different classification systems on the basis of individual laboratory predictions using the mean value of viability of complete run sequences

Table 4.4: *Accuracy values of the Validated Reference Method (EpiSkin™) calculated on the basis of the rules described above for (a) the EU DSD classification system ("EU DSD") and (b) the UN GHS classification system ("UN GHS"), for both the 58 SIVS chemicals as well as the 20 new RC.*

Individual Laboratory Predictions				
	58 SIVS chemicals		**20 R. Chemicals**	
	EU DSD	**UN GHS**	**EU DSD**	**UN GHS**
Specificity	82.1	70.0	79.2	78.6
Sensitivity	77.1	91.4	84.0	95.2
Overall Accuracy	80.0	74.6	81.6	85.7

The individual predictions of the VRM **for each chemical** in each laboratory underlying these recalculated accuracy values are shown in Tables 4.5 and 4.6.

Text Box 4.1: ***Note on the method of Predictive Capacity calculation:***

The accuracy values were calculated in agreement with common definitions:

Specificity = TN / (TN + FP)

Sensitivity = TP / (TP+FN)

Overall Accuracy = (TP + TN) / (TP+TN+FN+FP)

Where TN = True Negative, FP = False Positive, TP= True Positive, FN = False Negative.

The use of individual laboratory predictions for calculating predictive values is considered best practice. This method reflects best the real testing situation where decisions are made on the basis of date from one and not three laboratories. Therefore, predictive values should be based on individual laboratory predictions and not the mode of those.

Nota Bene:

1) The accuracy values published in the original ***ECVAM Performance Standards*** *and the* ***ESAC statement*** *pertaining to the SIVS were based on another slightly different calculation method using the median of the classifications resulting from the different runs for each chemical in each laboratory. The specificity was slightly lower (80.2%), while the sensitivity was as stated as above: 74.7%. The overall accuracy was slightly lower (78.2%). As outlined above, best practice is now the calculation on the basis of individual laboratory predictions, using the mean value of viability of all runs.*

b. Results of the recalculation

Upon recalculation of the accuracy values for the 58 SIVS chemicals, the VRM showed increased sensitivity (by about 14%) and decreased specificity (by about 12%) when comparing the EU DSD and the UN GHS classification systems (Table 4.4). This is the logical consequence of the threshold shift: with

substances being categorised irritant from a higher *in vivo* score on, test methods will tend to have a higher sensitivity.

When comparing the FN and FP rates of individual laboratory predictions (Tables 4.5 and 4.6), it becomes obvious, that the predictions of substances 34 to 45 are inversed: substances that were FN under the EU DSD system are correctly classified under UN GHS, while correctly classified substances under EU DSD become now FP under UN GHS.

4.2.2.2 Rationale for the new target values for Predictive Capacity in the Adapted Performance Standards

On the basis of the recalculated values under GHS, the target values for Predictive Capacity were chosen to be

Specificity =	**70 %**
Sensitivity =	**80 %**
Accuracy =	**75 %**

Please note that these values are derived from accuracy values based on all 58 SIVS chemicals and not only the 20 new Reference Chemicals (Table 4.4), because in the latter, there are 4 substances (cinnamaldehyde, potassium hydroxide 5%, tetrachloroethylene and heptanal) for which only data from a single laboratory exists (Table 4.3) and are thus underweighted in the calculations. Nevertheless, if one considers only the median of the individual laboratory predictions (final call) for the 20 new Reference Chemicals (as shown in Table 4.2) when calculating the Predictive Capacity of the VRM under UN GHS, the values obtained for sensitivity (90%), specificity (70%) and overall accuracy (80%) are similar to the ones obtained with the 58 SIVS chemicals (Table 4.4). However, in the case of the sensitivity, the chosen target value (80%) does not directly reflect the values obtained with the individual laboratory predictions for the 58 SIVS chemicals (91.4%) or with the final call for the 20 new RC (90%). The choice of 80% as target value for sensitivity in the Performance Standards was made taking into consideration additional information relating to relevance in the species of interest. Thus, since one of the 10 proposed GHS Cat 2 RC (di-n-propyl disulphide) is a possible false positive of the VRM and known to be non-irritant in humans (6/30 positive reactions in the human 4 h patch test) (Jirova et al. 2007), and another (1-decanol) is a UN GHS borderline chemical (in vivo score of 2.3) and also known to be non-irritant in humans (25/189 positive reactions on the human 4 h patch test) (Basketter et al. 2004; Jirova et al. 2007), it was decided to lower the target value for sensitivity from 90% to 80% but at the same time restrict possible misclassifications to these two chemicals only. Importantly, although the target value is lowered, the restrictions are actually higher, i.e. instead of allowing **one non-defined** GHS Cat 2 RC to be misclassified by a proposed equivalent RhE test method (target sensitivity at 90%), the revised PS allow for the misclassification of **two defined** GHS Cat 2 RC, i.e. di-n-propyl disulphide and 1-decanol.

It is also important to note that the sensitivity of the VRM calculated with the 58 SIVS substances for each of the three individual laboratories under UN GHS varies between 82% and 100% considering only complete run sequences and between 85% and 92% considering all runs. These numbers further support the appropriateness of setting the target value for sensitivity at 80%.

Laboratory	**Sensitivity**	
	Complete run sequences	**All runs**
L'Oréal	92% (11/12)	85% (11/13)
Unilever	100% (12/12)	92% (12/13)
Sanofi	82% (9/11)	85% (11/13)

The target value for specificity and overall accuracy directly reflect the values obtained with the individual laboratory predictions for the 58 SIVS chemicals, as well as with the final call for the 20 new RC. The target value for the overall accuracy (75%) is also in agreement with the overall accuracy required in the original PS under the EU DSD classification system. The different accuracy values of VRM including the new PS values are plotted in Figure 4.2.

In summary, the sensitivity should be equal or higher (≥) than 80%, but only 1-decanol and di-n-propyl disulphide (*in vivo* Category 2 substances) may be misclassified as "No Category" by more than one participating laboratory in an equivalence validation study. The specificity should be equal or higher (≥) than 70% with no further restriction applying, i.e. any participating laboratory may misclassify any *in vivo* No Category substance as long as the final specificity of the proposed RhE test method is within the acceptable range. The overall accuracy should be equal or higher (≥) than 75%.

Figure 4.2: *Accuracy values of VRM for the 2 classification systems (EU DSD and UN GHS) on the basis of the new calculation rules laid out in the revised PS. The suggested target values for the PS are plotted last: UN GHS (PS).*

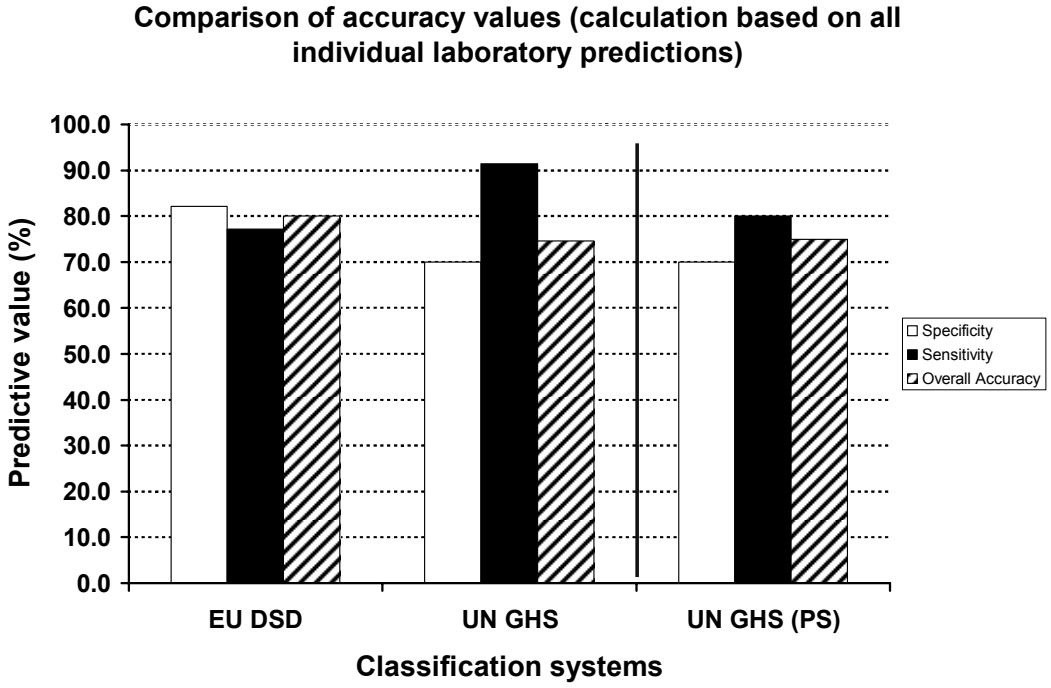


Table 4.5: *Individual laboratory predictions based on the EU Dangerous Substances Directive (DSD) classification system and using the validated 50% cut-off prediction model. Green = "non-irritant", pink = "irritant". Yellow = non-valid runs in SIVS (no data shown, depicted as n.a.). Red Line = in vivo threshold for distinguishing R38 from no-label substances in the EU DSD classification system. Dashed line = in vivo threshold for the GHS classification system. Substances included in the list of Reference Chemicals are colored in blue.*

				Classification			MTT mean of 3 valid runs			Decision		
count	SIVS Nr.	Substances Identities	Score	EU DSD	UN GHS*	UN GHS	L'Oréal	Unilever	Sanofi	Lab 1	Lab 2	Lab 3
1	2	1-bromo-4-chlorobutane	0	no label	no category	no category	5.95	4.02	4.40	1	1	1
2	6	3-diethylaminopropionitrile	0	no label	no category	no category	24.95	51.20	n.a.	1	0	n.a.
3	7	3-mercaptohexanol	0	no label	no category	no category	62.63	15.52	44.36	0	1	1
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	0	no label	no category	no category	114.34	109.48	101.48	0	0	0
5	22	diethyl phthalate	0	no label	no category	no category	95.31	75.04	92.54	0	0	0
6	24	di-propylene glycol	0	no label	no category	no category	106.24	93.56	97.44	0	0	0
7	25	dipropylene glycol monobutyl ether	0	no label	no category	no category	105.32	93.24	103.08	0	0	0
8	26	3,4-dimethyl-1H-pyrazole	0	no label	no category	no category	7.02	31.30	2.60	1	1	1
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine-1-carboxylate	0	no label	no category	no category	118.51	116.10	113.74	0	0	0
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	0	no label	no category	no category	99.72	111.79	102.26	0	0	0
11	35	cyclohexadecanone	0	no label	no category	no category	121.85	112.37	114.87	0	0	0
12	41	naphthalene acetic acid	0	no label	no category	no category	96.39	88.59	91.96	0	0	0
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	0	no label	no category	no category	100.30	101.39	95.25	0	0	0
14	48	2-(formylamino)-3-thiophenecarboxylic acid	0	no label	no category	no category	95.82	89.02	93.43	0	0	0
15	53	silane A-1430	0	no label	no category	no category	n.a.	n.a.	76.04	n.a.	n.a.	0
16	57	triethylene glycol	0	no label	no category	no category	97.97	100.56	101.49	0	0	0
17	9	2,6-dimethyl-4-nitrobenzeneamine	0.3	no label	no category	no category	107.13	98.58	104.36	0	0	0
18	11	allyl phenoxyacetate	0.3	no label	no category	no category	96.82	96.39	102.13	0	0	0
19	36	isopropanol	0.3	no label	no category	no category	100.41	80.46	86.96	0	0	0
20	12	2-ethylhexyl 4-aminobenzoate	0.7	no label	no category	no category	111.44	90.87	105.37	0	0	0
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0.7	no label	no category	no category	77.95	65.37	85.06	0	0	0
22	5	3-chloro-4-fluoronitrobenzene	1	no label	no category	no category	n.a.	10.96	10.17	n.a.	1	1
23	8	4-methylthio-benzaldehyde	1	no label	no category	no category	51.51	11.65	34.69	0	1	1
24	16	capryl-isostearate	1	no label	no category	no category	99.00	95.95	102.33	0	0	0
25	39	methyl stearate	1	no label	no category	no category	103.99	90.33	101.08	0	0	0
26	44	phenylethylalcohol	1	no label	no category	no category	92.55	77.80	95.99	0	0	0
27	30	Mixture of isomers: diethyl cis-1,4-cyclohexanedicarboxylate; diethyl trans-1,4-cyclohexanedicarboxylate	1.3	no label	no category	no category	81.90	67.70	99.92	0	0	0
28	54	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)	1.3	no label	no category	no category	66.43	40.40	55.86	0	1	0
29	10	allyl heptanoate	1.7	no label	opt. cat. 3	no category	101.12	99.53	101.34	0	0	0
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	1.7	no label	opt. cat. 3	no category	10.81	4.44	9.73	1	1	1
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; 5-endo-decylbicyclo[2.2.1]hept-2-ene	1.7	no label	opt. cat. 3	no category	103.47	107.00	104.22	0	0	0
32	33	heptyl butyrate	1.7	no label	opt. cat. 3	no category	103.99	102.32	111.54	0	0	0
33	50	2-phenylhexanenitrile	1.7	no label	opt. cat. 3	no category	116.18	93.77	112.93	0	0	0
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	2	R38	opt. cat. 3	no category	4.97	4.85	13.44	1	1	1
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol; 56% (1'R,2R) & 40%(1'R,2S) isomer	2	R38	opt. cat. 3	no category	11.09	6.20	9.01	1	1	1
36	31	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	2	R38	opt. cat. 3	no category	12.05	11.43	7.78	1	1	1
37	34	hexyl salicylate	2	R38	opt. cat. 3	no category	99.85	100.49	94.56	0	0	0
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	2	R38	opt. cat. 3	no category	53.55	31.32	46.94	0	1	1
39	46	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]decan-2-ol	2	R38	opt. cat. 3	no category	12.36	7.55	4.54	1	1	1
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	2	R38	opt. cat. 3	no category	14.82	31.50	13.33	1	1	1
41	49	isostearic acid monoisopropanolamide	2	R38	opt. cat. 3	no category	95.69	84.86	96.59	0	0	0
42	51	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)	2	R38	opt. cat. 3	no category	85.67	84.20	66.65	0	0	0
43	55	terpinyl acetate	2	R38	opt. cat. 3	no category	52.96	6.26	n.a.	0	1	n.a.
44	58	tri-isobutyl phosphate	2	R38	opt. cat. 3	no category	7.12	5.89	6.90	1	1	1
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	2	R38	opt. cat. 3	no category	88.36	6.35	78.81	0	1	0
46	4	1-decanol	2.3	R38	category 2	category 2	7.31	6.45	6.94	1	1	1
47	20	cyclamen aldehyde	2.3	R38	category 2	category 2	24.37	8.53	n.a.	1	1	n.a.
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	2.7	R38	category 2	category 2	5.67	4.66	3.92	1	1	1
49	3	1-bromohexane	2.7	R38	category 2	category 2	26.23	11.90	46.79	1	1	1
50	15	α-terpineol	2.7	R38	category 2	category 2	14.72	2.75	6.40	1	1	1
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	2.7	R38	category 2	category 2	11.71	9.79	8.83	1	1	1
52	18	butyl methacrylate	3	R38	category 2	category 2	11.25	10.65	n.a.	1	1	n.a.
53	23	di-n-propyl disulphide	3	R38	category 2	category 2	n.a.	7.49	77.01	n.a.	1	0
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	3	R38	category 2	category 2	9.15	9.62	11.09	1	1	1
55	40	1-methyl-3-phenyl-1-piperazine	3.3	R38	category 2	category 2	9.47	17.40	4.30	1	1	1
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	R38	category 2	category 2	12.74	n.a.	14.19	1	n.a.	1
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	4	R38	category 2	category 2	81.03	11.21	89.54	0	1	0
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	4	R38	category 2	category 2	21.91	14.76	17.12	1	1	1

Table 4.6: *Individual laboratory predictions based on the UN GHS classification system and using the validated 50% cut-off prediction model. Green = "non-irritant", pink = "irritant". Yellow = non-valid runs in SIVS (no data shown, depicted as n.a.). Red Line = in vivo threshold for distinguishing category 2 from no category substances under the UN GHS classification system as implemented in the EU. Dashed line = in vivo threshold for the EU DSD classification system. Substances included in the list of Reference Chemicals are coloured in blue.*

				Classification			MTT mean of 3 valid runs			Decision		
count	SIVS Nr.	Substances Identities	Score	EU DSD	UN GHS*	UN GHS	L'Oréal	Unilever	Sanofi	Lab 1	Lab 2	Lab 3
1	2	1-bromo-4-chlorobutane	0	no label	no category	no category	5.95	4.02	4.40	1	1	1
2	6	3-diethylaminopropionitrile	0	no label	no category	no category	24.95	51.20	n.a.	1	0	n.a.
3	7	3-mercaptohexanol	0	no label	no category	no category	62.63	15.52	44.36	0	1	1
4	19	2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate	0	no label	no category	no category	114.34	109.48	101.48	0	0	0
5	22	diethyl phthalate	0	no label	no category	no category	95.31	75.04	92.54	0	0	0
6	24	di-propylene glycol	0	no label	no category	no category	106.24	93.56	97.44	0	0	0
7	25	dipropylene glycol monobutyl ether	0	no label	no category	no category	105.32	93.24	103.08	0	0	0
8	26	3,4-dimethyl-1H-pyrazole	0	no label	no category	no category	7.02	31.30	2.60	1	1	1
9	28	ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine-1-carboxylate	0	no label	no category	no category	118.51	116.10	113.74	0	0	0
10	32	2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane	0	no label	no category	no category	99.72	111.79	102.26	0	0	0
11	35	cyclohexadecanone	0	no label	no category	no category	121.85	112.37	114.87	0	0	0
12	41	naphthalene acetic acid	0	no label	no category	no category	96.39	88.59	91.96	0	0	0
13	42	disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disulfonic acid or monosulfonic acid, monosulfonate or disulfonate	0	no label	no category	no category	100.30	101.39	95.25	0	0	0
14	48	2-(formylamino)-3-thiophenecarboxylic acid	0	no label	no category	no category	95.82	89.02	93.43	0	0	0
15	53	silane A-1430	0	no label	no category	no category	n.a.	n.a.	76.04	n.a.	n.a.	0
16	57	triethylene glycol	0	no label	no category	no category	97.97	100.56	101.49	0	0	0
17	9	2,6-dimethyl-4-nitrobenzeneamine	0.3	no label	no category	no category	107.13	98.58	104.36	0	0	0
18	11	allyl phenoxyacetate	0.3	no label	no category	no category	96.82	96.39	102.13	0	0	0
19	36	isopropanol	0.3	no label	no category	no category	100.41	80.46	86.96	0	0	0
20	12	2-ethylhexyl 4-aminobenzoate	0.7	no label	no category	no category	111.44	90.87	105.37	0	0	0
21	52	propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate	0.7	no label	no category	no category	77.95	65.37	85.06	0	0	0
22	5	3-chloro-4-fluoronitrobenzene	1	no label	no category	no category	n.a.	10.96	10.17	n.a.	1	1
23	8	4-methylthio-benzaldehyde	1	no label	no category	no category	51.51	11.65	34.69	0	1	1
24	16	capryl-isostearate	1	no label	no category	no category	99.00	95.95	102.33	0	0	0
25	39	methyl stearate	1	no label	no category	no category	103.99	90.33	101.08	0	0	0
26	44	phenylethylalcohol	1	no label	no category	no category	92.55	77.80	95.99	0	0	0
27	30	Mixture of isomers: diethyl cis-1,4-cyclohexanedicarboxylate; diethyl trans-1,4-cyclohexanedicarboxylate	1.3	no label	no category	no category	81.90	67.70	99.92	0	0	0
28	54	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)	1.3	no label	no category	no category	66.43	40.40	55.86	0	1	0
29	10	allyl heptanoate	1.7	no label	opt. cat. 3	no category	101.12	99.53	101.34	0	0	0
30	17	2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer	1.7	no label	opt. cat. 3	no category	10.81	4.44	9.73	1	1	1
31	21	A mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; 5-endo-decylbicyclo[2.2.1]hept-2-ene	1.7	no label	opt. cat. 3	no category	103.47	107.00	104.22	0	0	0
32	33	heptyl butyrate	1.7	no label	opt. cat. 3	no category	103.99	102.32	111.54	0	0	0
33	50	2-phenylhexanenitrile	1.7	no label	opt. cat. 3	no category	116.18	93.77	112.93	0	0	0
34	13	1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one	2	R38	opt. cat. 3	no category	4.97	4.85	13.44	1	1	1
35	29	Mixture of: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol; 56% (1'R,2R) & 40%(1'R,2S) isomer	2	R38	opt. cat. 3	no category	11.09	6.20	9.01	1	1	1
36	31	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	2	R38	opt. cat. 3	no category	12.05	11.43	7.78	1	1	1
37	34	hexyl salicylate	2	R38	opt. cat. 3	no category	99.85	100.49	94.56	0	0	0
38	43	A mixture of isomers: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane	2	R38	opt. cat. 3	no category	53.55	31.32	46.94	0	1	1
39	46	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]decan-2-ol	2	R38	opt. cat. 3	no category	12.36	7.55	4.54	1	1	1
40	47	4-methyl-8-methylenetricyclo[3.3.1.1(3,7)]dec-2-yl acetate	2	R38	opt. cat. 3	no category	14.82	31.50	13.33	1	1	1
41	49	isostearic acid monoisopropanolamide	2	R38	opt. cat. 3	no category	95.69	84.86	96.59	0	0	0
42	51	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)	2	R38	opt. cat. 3	no category	85.67	84.20	66.65	0	0	0
43	55	terpinyl acetate	2	R38	opt. cat. 3	no category	52.96	6.26	n.a.	0	1	n.a.
44	58	tri-isobutyl phosphate	2	R38	opt. cat. 3	no category	7.12	5.89	6.90	1	1	1
45	60	bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex	2	R38	opt. cat. 3	no category	88.36	6.35	78.81	0	1	0
46	4	1-decanol	2.3	R38	category 2	category 2	7.31	6.45	6.94	1	1	1
47	20	cyclamen aldehyde	2.3	R38	category 2	category 2	24.37	8.53	n.a.	1	1	n.a.
48	1	2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	2.7	R38	category 2	category 2	5.67	4.66	3.92	1	1	1
49	3	1-bromohexane	2.7	R38	category 2	category 2	26.23	11.90	46.79	1	1	1
50	15	α-terpineol	2.7	R38	category 2	category 2	14.72	2.75	6.40	1	1	1
51	45	(+/-) trans-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol	2.7	R38	category 2	category 2	11.71	9.79	8.83	1	1	1
52	18	butyl methacrylate	3	R38	category 2	category 2	11.25	10.65	n.a.	1	1	n.a.
53	23	di-n-propyl disulphide	3	R38	category 2	category 2	n.a.	7.49	77.01	n.a.	1	0
54	37	[2-(cyclopentyloxy)ethyl]benzene(cyclopentyl 2-phenylethyl ether)	3	R38	category 2	category 2	9.15	9.62	11.09	1	1	1
55	40	1-methyl-3-phenyl-1-piperazine	3.3	R38	category 2	category 2	9.47	17.40	4.30	1	1	1
56	56	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	R38	category 2	category 2	12.74	n.a.	14.19	1	n.a.	1
57	27	2-isopropyl-2-isobutyl-1,3-dimethoxypropane	4	R38	category 2	category 2	81.03	11.21	89.54	0	1	0
58	59	(E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol	4	R38	category 2	category 2	21.91	14.76	17.12	1	1	1

4.3 Calculation of *In Vivo* Scores Under UN GHS

The recalculations of Predictive Capacity for the VRM under the UN GHS classification system, as presented in the previous chapters, were done by comparing the in vitro predictions to in vivo classifications obtained by considering an in vivo dominant median value of 2.3 or above for classifying a substance as GHS Category 2, as opposed to a score of 2.0 under the EU DSD classification system. The in vivo dominant median values for each tested substance were calculated as previously done during the Skin Irritation Validation Study (SIVS), when considering the EU DSD classification system, i.e. the average score over 24, 48 and 72 hours for oedema and erythema was calculated for each animal, the median of the average scores of all animals was calculated for oedema and erythema, and the highest value of oedema or erythema was taken as the in vivo dominant score.

Although the EU CLP/UN GHS and EU DSD classification systems differ in other criteria beside the classification cut-off (2.3 vs. 2.0), for distinguishing non-classified from classified substances, such as irreversibility in at least two animals and pronounced effects in a single animal, none of the 58 SIVS chemicals or new RC was classified based on any of these criteria alone. All chemicals that showed non reversible or pronounced effects in a single animal were classified as GHS Cat 2 based on the dominant median of mean scores of 24, 48 and 72 hour readings as well.

Moreover, the CLP, UN Purple Book and Annex V of Directive 67/548 all give similar guidance in relation to scoring where 3 animals have been used (which will be the norm in any new test), using a 2 out of 3 rule:

Annex V

"In the case where the Annex V test has been completed using three animals, either erythema, eschar formation or oedema formation equivalent to a mean value of 2 or more calculated for each animal separately has been observed in two or more animals."

EU CLP

"Mean value of 2.3 to 4 for erythema/eschar in at least 2 of 3 animals tested from grading at 24, 48 and 72 hours after patch removal....."

Importantly, the dominant median calculation expresses the exact same outcome as the 2 out of 3 rule (e.g. for the worst case scenario of mean scores of readings at 24, 48 and 72 hours of 2.3, 2.3, and 0 for three animals, the median (2.3) would express the same result as 2 out of 3 animals).

On the other hand, there are no criteria in the UN GHS for tests with more then 3 animals (a considerable proportion of the 58 SIVS chemicals). While guidance exists in Annex V for these cases, none is given in EU CLP. However, the agreed but not yet adopted EU RIP 3.6: Guidance on Classification and Labeling under Global Harmonized System, acknowledges that specific provisions need to be applied in cases where substances were tested in more than three animals, and includes guidance on how to handle classification for such skin irritation studies (chapter *"Tests that have been conducted with more than three animals"*):

RIP 3.6

"For the sake of flexibility basically two approaches can be accepted for evaluation**:**

- the overall average over all animals will be used (see Example 3a). This has been common practice under the DSD.
- According to the second approach the average score is determined per animal (see Example 3b). In this case Skin Irritant Category 2 is assigned if 4 of 6 rabbits show a mean score of 2.3 or above. Likewise, if the test was performed with 4 or 5 animals, for at least 3 individuals the mean score must exceed the value of 2.3 to classify as Skin Irritant Category 2.

The more stringent result has to be used if the evaluation according to the method shown under Example 3a is different to that under Example 3b."

In summary, the two approaches recommended in RIP 3.6 are (i) to use the overall average over all tested animals, and (ii) apply a variation of the 2 out of 3 rule (classify as GHS Cat 2 if 67% of the tested animals show mean scores of readings at 24, 48 and 72 hours ≥ 2.3), where the most conservative outcome should be used to derive the classification. Although, both the EU DSD and RIP 3.6 recommend using the mean (=average) of all animals to derive the classification, the median is usually used instead if producing a more conservative outcome. As a practical example, a substance with scores from 6 rabbits of 2.0, 2.0, 2.0, 2.0, 2.0 and 1.7 would be classified as R38 based on median under the EU DSD classification system, even though the mean score would be below 2.0.

The following calculations to derive final classifications for the 58 SIVS chemicals under UN GHS were performed:

Classify a substance as GHS Cat 2 if:

1) the **mean** of all mean rabbit scores is ≥ 2.3 and, if the mean of all mean rabbit scores is < 2.3 and only three animals were tested, the individual mean rabbit scores of 2 out of 3 rabbits are ≥ 2.3
2) the **median** of all mean rabbit scores is ≥ 2.3 and, if the median of all mean rabbit scores is < 2.3 and only three animals were tested, the individual mean rabbit scores of 2 out of 3 rabbits are ≥ 2.3
3) **67%** of the individual mean rabbit scores are ≥ 2.3

The results showed that the most stringent classification is obtained from option 2, giving the exact same final classifications as obtained by simply changing the cut-off from 2.0 to 2.3. A combination of options 1 and 3 as strictly specified in RIP 3.6 originates a different outcome than option 2 alone (or in combination with option 3 - same final result obtained) for one single substance, cyclamen aldehyde, where options 1 + 3 lead to a GHS Cat 3 categorization, whereas option 2 results in a GHS Cat 2 categorization. Such discrepancy originates from oedema scores of 3.0, 3.0, 2.7, 2.3, 2.7, 2.0, 1.7, 2.7, 2.7, 2.3, 3.0, 1.3, 1.0, 2.0, and 1.3. The average of these 15 scores is 2.247, the median is 2.3 and only 9 animals have scores ≥ 2.3 (67% is 10 animals). In this case, the median provides the most conservative outcome and should thus be used. Similarly, under the EU DSD classification system the SIVS substance terpinyl acetate was classified R38 with erythema scores of 1.7, 2.0, 2.0, 2.0, 2.0, 2.0, 2.0, 2.0, 1.7, 2.0, 1.3 (average = 1.882; median = 2.0), and with oedema scores of 1.0, 2.0, 2.0, 3.0, 2.0, 2.3, 2.0, 2.0, 1.0, 0.7, 0.3 (average = 1.664; median = 2.0), although for both endpoints the average of the rabbit mean scores of readings at 24, 48 and 72 hours was less than 2.0. Importantly, all *in vivo* EU DSD classifications used in SIVS were verified and approved by several EU regulators.

In conclusion, the *in vivo* classifications used for the recalculation with respect to the UN GHS classification system are correct and consequently the stated test method performances are adequate.

5. INFORMATION FROM OPTIMISATION STUDIES

Prior to the SIVS validation, both the EpiSkin (section 5.1) and the original EpiDerm (section 5.2) underwent extensive testing following optimisation of their protocols. The test substances were based on the chemicals listed in the ECETOC database Nr. 66 on skin irritation chemicals (**Table 5.3**). In addition, three other chemicals were tested (Table 5d) in the EpiSkin, but not the original EpiDerm. The results of these optimisation studies have been published in 2005 by Cotovio et al. (EpiSkin; Annex 1 doc20) and by Kandarova et al. (EpiDerm; Annex 1 doc19). The chemical structure of the test substances used in the optimisation studies are provided in **Annexe 9**.

The predictive capacity of the two optimised protocols during the optimisation study is summarised in **Table 5.1** (EpiSkin) and **Table 5.2** (original EpiDerm). Note that the protocols used in the testing studies following optimisation were also used in the SIVS.

Table 5.1: *EpiSkin test method - contingency table of the predictions obtained during the optimisation study. Red: False Negative (FN) predictions in vitro. Blue: False Positive (FP) predictions in vitro.*

EpiSkin test method (optimisation study)

EU DSD

		in vitro I	NI	Σ
in vivo	I	**16**	**3**	19
	NI	**6**	**20**	26
	Σ	22	23	***45***

Sensitivity (%)	**84.2**
Specificity (%)	**76.9**
Accuracy (%)	**80.0**

UN GHS

		in vitro I	NI	Σ
in vivo	I	**9**	**1**	10
	NI	**13**	**22**	35
	Σ	22	23	***45***

Sensitivity (%)	**90.0**
Specificity (%)	**62.9**
Accuracy (%)	**68.9**

Table 5.2: *original EpiDerm test method - contingency table of the predictions obtained during the optimisation study. Red: False Negative (FN) predictions in vitro. Blue: False Positive (FP) predictions in vitro.*

Original EpiDerm test method (optimisation study)

EU DSD

		in vitro I	NI	Σ
in vivo	I	**13**	**6**	19
	NI	**4**	**22**	26
	Σ	17	28	***45***

Sensitivity (%)	**68.4**
Specificity (%)	**84.6**
Accuracy (%)	**77.8**

UN GHS

		in vitro I	NI	Σ
in vivo	I	**7**	**3**	10
	NI	**10**	**25**	35
	Σ	17	28	***45***

Sensitivity (%)	**70.0**
Specificity (%)	**71.4**
Accuracy (%)	**71.1**

Table 5.3: Test chemicals used in the two optimisation studies pertaining to the EpiSkin and the original EpiDerm assay. Individual erythema and oedema scores computed for each animal and in vivo experiment listed in ECETOC database no. 66.

Count	No	Chemical	Ind. scores for erytheme in Animal No.						Median	Ind. scores for oedeme in Animal No						Median	Dom. Median[1]	Classification		
			1	2	3	4	5	6		1	2	3	4	5	6			EU DSD	UN GHS*	UN GHS
1	3	3.3-Dithiodipropionic acid	0	0	0	0			0	0	0	0	0			0	0.0	No	No	No
2	4	3-Chloronitrobenzene	0	0	0				0	0	0	0				0	0.0	No	No	No
3	5	4.4-Methylene-bis (2.6-ditertbutyl)phenol	0	0	0				0	0	0	0				0	0.0	No	No	No
4	6	4-Amino-1.2.4-triazole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	No	No	No
5	12	Benzyl benzoate - I	0	0	0				0	0	0	0				0	0.0	No	No	No
		Benzyl benzoate - II	0	1.7	1	2				0	0.3	0.3	1							
6	15	Dipropylene glycol - I	0	0	1				0	0	0	0				0	0.0	No	No	No
		Dipropylene glycol - II	0	0	0	0			0	0	0	0	0							
7	16	Erucamide	0	0	0				0	0	0	0				0	0.0	No	No	No
8	24	Sodium bicarbonate	0.3	0	0				0	0	0	0				0	0.0	No	No	No
9	13	Benzyl salicylate - I	0.3	0.7	0				0.3	0	0	0				0	0.3	No	No	No
		Benzyl salicylate - II	1	0.3	1	0				0.3	0	0	0.3							
10	18	Isopropanol	1.7	0.3	0.3				0.3	0	0	0				0	0.3	No	No	No
11	21	Lauric acid	1	0	0.3				0.3	0	0	0				0	0.3	No	No	No
12	1	2.4-Xylidine	1	1	1				1	1	1	0				1	1	No	No	No
13	8	Soap from 20/80 coconut oil/tallow	1	1	1				1	1	1	1				1	1	No	No	No
14	9	1.6 - Dibromohexane	0.7	1	1				1	0	0	0				0	1	No	No	No
15	11	Benzyl acetate - I	1.7	0	1.3				1	1	0	0.7				0	1	No	No	No
		Benzyl acetate - II	1.7	0	0.7	1				0	0	0	0							
16	17	Hydroxycitronellal - I	1	1	0.7				1	0.3	0.3	0				0	1	No	No	No
		Hydroxycitronellal - II	1.7	0	0.7	1				0.3	0	0	0							
17	19	Isopropyl myristate	1	1	1				1	0.7	0	0				0	1	No	No	No
18	20	Isopropyl palmitate	1	1.7	1				1	0	0.7	0				0	1	No	No	No
19	22	Methylstearate	1	2.3	1				1	0	2.0	0				0	1	No	No	No
20	23	n-Butyl propionate	1.7	0.7	1	1			1	0	0	0	0			0	1	No	No	No
21	25	Sodium bisulphite	1	1	1				1	0	0	0				0	1	No	No	No
22	14	Benzylalcohol - I	1.7	1	1.3				1.3	0.3	0	0.3				0.3	1.3	No	No	No
		Benzylalcohol - II	1	1.7	1	2.0				0	0.7	0.3	0.7							

Count	No	Chemical	Ind. scores for erytheme in Animal No.						Median	Ind. scores for oedeme in Animal No						Median	Dom. Median[1]	Classification		
			1	2	3	4	5	6		1	2	3	4	5	6			EU DSD	UN GHS*	UN GHS
23	2	2-Methyl-4-phenyl-2-butanol	1.7	1.7	1	1			1.4	1	0.3	0.3	0.7			0.5	1.4	No	No	No
24	7	cis-Cyclooctene	2	1	2	1.7	1	1.3	1.5	0.7	0	0.7	0.7	0	0.3	0.5	1.5	No	No	No
25	10	2-ethoxy ethyl methacrylate	1	1.7	2				1.7	0	0	0				0	1.7	No	Opt. cat. 3	No
26	45	Tallow propylene polyamine I	2	0.7	2				1.7	1.3	0	2				1.7	1.7	No	Opt. cat. 3	No
		Tallow propylene polyamine II	1.3	3.7	0					3.7	3.7	0								
27	27	10-Undecenoic acid	2	2	2	2			2	1	1.3	1	0.3			1	**2**	**R38**	Opt. cat. 3	No
28	29	dl-Citronellol - I	2	2	2				2	2	2.7	2				2	**2**	**R38**	Opt. cat. 3	No
		dl-Citronellol - II	2	2	2	2				2.7	2.7	1.3	1.3							
		dl-Citronellol - II	2	2	2	2				2	2	1.3	1.3							
29	30	d-Limonene - I	2	2	2				2	1.3	2	1.3				1.3	**2**	**R38**	Opt. cat. 3	No
		d-Limonene - I	2	2	1.7	2				1.3	0.7	1.3	2							
30	32	Lilestralis/lilial - I	1.7	2	2.3				2	2	2.7	3				2	**2**	**R38**	Opt. cat. 3	**No**
		Lilestralis/lilial - II	2	1.7	2	2				1.7	1.7	2.3	1							
31	39	Cinnamaldehyde	2	2	2	2.3			2	2	2	1.3	2.3			2	**2**	**R38**	Opt. cat. 3	**No**
32	40	Eugenol	2	2	2	2			2	1.7	1.3	1	1			1.2	**2**	**R38**	Opt. cat. 3	**No**
33	41	Linalol - I	2	2	1.7				2	2	1.3	1				1	**2**	**R38**	Opt. cat. 3	**No**
		Linalol - II	2	2	2.0	2				1.7	1.7	1	1.3							
		Linalol - III	1	2	2	1.7				0	1	0.7	0							
34	42	Linalyl acetate - I	1.7	2	2				2	1.7	2	2				2	**2**	**R38**	Opt. cat. 3	**No**
		Linalyl acetate - II	2	2	2	1.7				2	2	2	1.7							
35	43	Methyl laurate	2	2	2				2	2	2	2				2	**2**	**R38**	Opt. cat. 3	**No**
36	28	1-Brompentane	3.7	1.7	2.7				2.7	2.7	0	2.7				2.7	2.7	R38	Cat. 2	Cat. 2
37	37	1-Bromohexane	2.7	2	2.7				2.7	0	0.7	2				0.7	2.7	R38	Cat. 2	Cat. 2
38	38	alpha-Terpineol - I	1.7	2	1.3				2	2	2.3	3				2.7	2.7	R38	Cat. 2	Cat. 2
		alpha-Terpineol - II	2	2.7	2	2				3	3	2.7	1.7						Cat. 2	Cat. 2

Count	No	Chemical	Ind. scores for erytheme in Animal No.						Median	Ind. scores for oedeme in Animal No						Median	Dom. Median[1]	Classification		
			1	2	3	4	5	6		1	2	3	4	5	6			EU DSD	UN GHS*	UN GHS
		alpha-Terpineol - III	2	2	1.7	2				2.7	2	0.7	3						Cat. 2	Cat. 2
39	33	Methylpalmitate	1.5	3	3				3	1	2.5	2.5				2.5	3	R38	Cat. 2	Cat. 2
40	34	Potassium hydroxide (5% aq)	3	3	3.7				3	2	2	2				2	3	R38	Cat. 2	Cat. 2
41	31	Heptanal	4	4	2.7	2.3			3.4	2	2.3	3	2			2.2	3.4	R38	Cat. 2	Cat. 2
42	26	1.1.1-Trichloroethane	4	4	3				4	2	1.3	1.3				1.3	4	R38	Cat. 2	Cat. 2
43	35	Tetrachloroethylene	4	4	4				4	2	1.7	1.3				1.7	4	R38	Cat. 2	Cat. 2
44	36	SLS (50% aq.)	4	4	3.7				4	2	2	2.3				2	4	R38	Cat. 2	Cat. 2
45	44	SLS (20% aq.)	3.3	4	4				4	2.3	3	3.7				3	4	R38	Cat. 2	Cat. 2

5.1 Individual chemical data from the optimisation study of the EpiSkin method

Table 5.4: EpiSkin test method – *median (mode) of individual laboratory predictions based on the EU DSD system and UN GHS for the optimisation study.*

Nr.	No	Chemical	Cas-No	Score	Classification			Median	EU DSD	UN GHS
					EU DSD	UN GHS*	UN GHS			
1	**43**	3,3-Dithiodipropionic acid	1119-62-6	0	No	No	No	0	**TN**	**TN**
2	**46**	3-Chloronitrobenzene	121-73-3	0	No	No	No	0	**TN**	**TN**
3	**44**	4,4-Methylene-bis (2,6-di-butyl) phenol	118-82-1	0	No	No	No	0	**TN**	**TN**
4	**45**	4-Amino-1,2,4-triazole	584-13-4	0	No	No	No	0	**TN**	**TN**
5	**27**	Benzyl benzoate	120-51-4	0	No	No	No	0	**TN**	**TN**
6	**41**	Dipropylene glycol	25265-71-8	0	No	No	No	0	**TN**	**TN**
7	**48**	Erucamide	112-84-5	0	No	No	No	0	**TN**	**TN**
8	**42**	Sodium bicarbonate	144-55-8	0	No	No	No	0	**TN**	**TN**
9	**39**	Benzyl salicylate	118-58-1	0,3	No	No	No	0	**TN**	**TN**
10	**38**	Isopropanol	67-63-0	0,3	No	No	No	0	**TN**	**TN**
11	**40**	Lauric acid	143-07-7	0,3	No	No	No	0	**TN**	**TN**
12	**31**	2,4-Xylidine	95-68-1	1	No	No	No	1	**FP**	**FP**
13	**22**	Soap from 20/80 coconut oil/tallow	ND	1	No	No	No	0	**TN**	**TN**
14	**37**	1,6 Dibromohexane	629-03-8	1	No	No	No	1	**FP**	**FP**
15	**29**	Benzyl acetate	140-11-4	1	No	No	No	1	**FP**	**FP**
16	**34**	Hydroxycitronellal	107-75-5	1	No	No	No	0	**TN**	**TN**
17	**33**	Isopropyl myristate	110-27-0	1	No	No	No	0	**TN**	**TN**
18	**30**	Isopropyl palmitate	142-91-6	1	No	No	No	0	**TN**	**TN**
19	**23**	Methyl stearate	112-61-8	1	No	No	No	0	**TN**	**TN**
20	**35**	n-Butyl propionate	590-01-2	1	No	No	No	0	**TN**	**TN**
21	**36**	Sodium bisulphite	7775-14-6	1	No	No	No	0	**TN**	**TN**
22	**24**	Benzyl alcohol	100-51-6	1,3	No	No	No	0	**TN**	**TN**
23	**28**	2-Methyl-4-phenyl-2- butanol	130-05-9	1,4	No	No	No	1	**FP**	**FP**
24	**25**	*Cis*-Cyclooctene	931-87-3	1,5	No	No	No	1	**FP**	**FP**
25	**26**	2-ethoxy ethyl methacrylate	2370-63-0	1,7	No	Opt cat 3	No	0	**TN**	**TN**
26	**12**	Tallow polypropylene polyamine	68911-79-5	1,7	No	Opt cat 3	No	1	**FP**	**FP**
27	**19**	10-Undecenoic acid	112-38-9	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
28	**11**	dl-Citronellol	106-22-9	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
29	**17**	d-Limonene	5989-27-5	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
30	**7**	Lilestralis/lilial	80-54-6	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
31	**15**	Cinnamaldehyde	104-55-2	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
32	**18**	Eugenol	97-53-0	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
33	**20**	Linalol	78-70-6	2	**R38**	Opt cat 3	No	1	**TP**	**FP**
34	**16**	Linalyl acetate	115-95-7	2	**R38**	Opt cat 3	No	0	**FN**	**TN**
35	**14**	Methyl laurate	111-82-0	2	**R38**	Opt cat 3	No	0	**FN**	**TN**
36	**9**	1-Bromopentane	110-53-2	2,7	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
37	**13**	1-Bromohexane	111-25-1	2,7	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
38	**10**	Alpha terpineol	98-55-5	2,7	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
39	**8**	Methyl palmitate	112-39-0	3	**R38**	Cat. 2	Cat. 2	0	**FN**	**FN**
40	**5**	Potassium hydroxide 5%	1310-58-3	3	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
41	**6**	Heptanal	111-71-7	3,4	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
42	**4**	1,1,1 trichloroethane	71-55-6	4	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
43	**3**	Tetrachloroethylene	127-18-4	4	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
44	**2**	SLS 50% (aq,)	151-21-3	4	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**
45	**1**	SLS 20% (aq,)	151-21-3	4	**R38**	Cat. 2	Cat. 2	1	**TP**	**TP**

Green = non-irritant; violet = irritant ; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system (dashed line indicates the shift in threshold from EU DSD to UN GHS.

5.2 Individual chemical data from the optimisation study (original EpiDerm)

Table 5.5: EpiDerm test method – *median (mode) of individual laboratory predictions based on the EU DSD system and UN GHS for the optimisation study.*

Count	No.	Chemical	Score	Classification			Prediction[1]	Mode	EU DSD	UN GHS
				ECS	GHS	GHS EU				
1	3	3.3'-Dithiodipropionic acid	0	No	No	No	95.7	0	**TN**	**TN**
2	4	3-Chloronitrobenzene	0	No	No	No	96.9	0	**TN**	**TN**
3	5	4.4-Methylene bis-(2.6-di - *tert*-butyl) phenol	0	No	No	No	94.4	0	**TN**	**TN**
4	6	4-Amino-1.2.4-triazole	0	No	No	No	92.1	0	**TN**	**TN**
5	14	Benzyl alcohol	1.3	No	No	No	89.2	0	**TN**	**TN**
6	15	Dipropylene glycol	0	No	No	No	107.4	0	**TN**	**TN**
7	16	Erucamide	0	No	No	No	103.6	0	**TN**	**TN**
8	24	Sodium bicarbonate	0	No	No	No	100.6	0	**TN**	**TN**
9	12	Benzyl benzoate	0	No	No	No	100.1	0	**TN**	**TN**
10	18	Isopropanol	0.3	No	No	No	99.9	0	**TN**	**TN**
11	21	Lauric acid	0.3	No	No	No	101.4	0	**TN**	**TN**
12	1	2.4-Xylidine	1	No	No	No	13.3	1	**FP**	**FP**
13	8	Soap 20/80 coconut oil/tallow	1	No	No	No	96.6	0	**TN**	**TN**
14	9	1.6 - Dibromohexane	1	No	No	No	12.1	1	**FP**	**FP**
15	11	Benzyl acetate	1	No	No	No	102.8	0	**TN**	**TN**
16	17	Hydroxycitronellal	1	No	No	No	72.5	0	**TN**	**TN**
17	19	Isopropyl myristate	1	No	No	No	100.8	0	**TN**	**TN**
18	20	Isopropyl palmitate	1	No	No	No	107.8	0	**TN**	**TN**
19	22	Methylstearate	1	No	No	No	101	0	**TN**	**TN**
20	23	n-Butyl propionate	1	No	No	No	108.7	0	**TN**	**TN**
21	25	Sodium bisulphite	1	No	No	No	93.5	0	**TN**	**TN**
22	13	Benzyl salicylate	0.3	No	No	No	97.3	0	**TN**	**TN**
23	2	2-Methyl-4-phenyl-2-butanol	1.4	No	No	No	30.2	1	**FP**	**FP**
24	7	*Cis*-Cyclooctene	1.5	No	No	No	84.4	0	**TN**	**TN**
25	10	2-ethoxy ethyl methacrylate	1.7	No	Opt cat 3	No	99	0	**TN**	**TN**
26	45	Tallow propylene polyamine	1.7	No	Opt cat 3	No	20.6	1	**FP**	**FP**
27	27	10-Undecenoic acid	2	**R38**	Opt cat 3	No	13.2	1	**TN**	**FP**
28	29	*dl*-Citronellol	2	**R38**	Opt cat 3	No	11.1	1	**TN**	**FP**
29	30	d-Limonene	2	**R38**	Opt cat 3	No	16.3	1	**TN**	**FP**
30	32	Lilestralis/Lilial	2	**R38**	Opt cat 3	No	12	1	**TN**	**FP**
31	39	Cinnamaldehyde	2	**R38**	Opt cat 3	No	11.4	1	**TN**	**FP**
32	40	Eugenol	2	**R38**	Opt cat 3	No	11.8	1	**TN**	**FP**
33	41	Linalol	2	**R38**	Opt cat 3	No	73.1	0	**FN**	**TN**
34	42	Linalyl acetate	2	**R38**	Opt cat 3	No	95	0	**FN**	**TN**
35	43	Methyl laurate	2	**R38**	Opt cat 3	No	103.3	0	**FN**	**TN**
36	28	1-Bromopentane	2.7	**R38**	Cat. 2	Cat. 2	81.7	0	**FN**	**FN**
37	37	1-Bromohexane	2.7	**R38**	Cat. 2	Cat. 2	93.5	0	**FN**	**FN**
38	38	alpha-Terpinelol	2.7	**R38**	Cat. 2	Cat. 2	10.2	1	**TP**	**TP**
39	33	Methyl palmitate	3	**R38**	Cat. 2	Cat. 2	95.7	0	**FN**	**FN**
40	34	Potassium hydroxide (5%)	3	**R38**	Cat. 2	Cat. 2	10.5	1	**TP**	**TP**
41	31	Heptanal	3.4	**R38**	Cat. 2	Cat. 2	10.8	1	**TP**	**TP**
42	26	1.1.1-Trichloroethane	4	**R38**	Cat. 2	Cat. 2	16.2	1	**TP**	**TP**
43	35	Tetrachloroethylene	4	**R38**	Cat. 2	Cat. 2	14	1	**TP**	**TP**
44	36	SLS (50%)	4	**R38**	Cat. 2	Cat. 2	11.9	1	**TP**	**TP**
45	44	SLS (20% aq.)	4	**R38**	Cat. 2	Cat. 2	13.2	1	**TP**	**TP**

Green = non-irritant; violet = irritant ; TN = True Negative (Negative Test Result for a Substance that is an Actual Negative), TP = True Positive (Positive Test Result for a Substance that is an Actual Positive). FP = False Positive (Positive Test Result for a Substance that is an Actual Negative); FN: False Negative (Negative Test Result for Substance that is an Actual Positive); Red line = in vivo threshold EU DSD and UN GHS system (dashed line indicates the shift in threshold from EU DSD to UN GHS.

Table: *Test chemicals – additionally tested by Cotovio et al. (2005) but not by Kandarova et al (2005)*

47	Polyether siloxane	ND
32	Sodium metasilicate (10%)	6834-92-0
21	Dimethyl disulphide	624-92-0

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7. ABBREVIATIONS USED IN THIS DOCUMENT

ECVAM	European Centre for the Validation of Alternative Methods (at the Institute of Health and Consumer Protection, IHCP), European Commission Joint Research Centre.
EU DSD	European Classification System based on the Dangerous Substance Directive (DSD)
GHS	Globally Harmonised System on the Classification and Labelling of Chemicals
MTT	3-(4,5-Dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide. A vital dye used to assess cell viability via a colorimetric assay. Cell viability is used as a proxy for predicting the skin irritancy potential of xenobiotic substances in human reconstructed epidermis models.
PM	Prediction Model
PS	Performance Standards
RC	Reference Chemicals
SIVS	ECVAM Skin Irritation Validation Study
UN GHS	The GHS system for skin irritation as applicable to all authorities, i.e. using one irritant category.
UN GHS*	The GHS system for skin irritation using two irritant categories (including one optional additional mild category, only applicable to some authorities).

ANNEXE 1 – LIST AND SHORT DESCRIPTION OF 28 DOCUMENTS MADE AVAILABLE IN THE CONTEXT OF THE OECD EXPERT CONSULTATION

Nr.	File Name	Description
1	doc-01_2006-10-04_SIVS Summary Report Final.doc	Summary report of the outcome of the SIVS
2	doc-02_SIVS_Projectplan_04-01-22.pdf	Validation project plan pertaining to the SIVS
3	doc-03_EPISKIN-SIT-SOP 2007.pdf	EpiSkin SOP for use in the SIVS
4	doc-04_EpiDerm-SIT-SOP5.0_final.pdf	EpiDerm SOP for use in the SIVS
5	doc-05_SIFT Protocol Dec 2003.doc	Protocol of the Skin Integrity Function Test (SIFT) which was evacuate during the ECVAM prevalidation study (1999-2000)
6	doc-06_SIVS Phase II Stat Report final 310706 amended 140906 corr..pdf	Statistical report of the results of phase II of the SIVS. Phase II was the actual testing phase involving the blind testing of 58 carefully selected chemicals per test and concerned the EpiSkin and EpiDerm methods. The preceding Phase I was dedicated to the evaluation of the refinements made as a result of the prevalidation study. Phase I encompassed the EpiSkin, EpiDerm and SIFT test. Only the EpiDerm and EpiSkin assays proceeded to Phase II.
7	doc-07_Report_CSSC_&_ Appendix 1-final.pdf	Report of the Chemical Selection Subcommittee of the SIVS concerning possible reasons for the misclassifications obtained with the EpiSkin and EpiDerm methods during the SIVS
8	doc-08_Appendix 2 to CSSC Report_06-09-27.pdf	Report on the application of the BfR (Bundesinstitut für Risikobewertung) Rulebase to the test substances used in the SIVS
9	doc-09_SIVS_ Performance Standards_ final.pdf	Original ECVAM Performance Standards as of May 2007. The modified EpiDerm and the SkinEthic assays were validated in relation to these Performance Standards.
10	doc-10_SIVS_EPISKIN_ Phase1_04-11-03.PDF	Report on the results obtained for the EpiSkin method during Phase I of the SIVS.
11	doc-11_SIVS_EPiDerm_Phase1_04-08-24.pdf	Report on the results obtained for the EpiDerm method during Phase I of the SIVS.
12	doc-12_SIVS_SIFT_Phase1_04-10-28.pdf	Report on the results obtained for the SIFT method during Phase I of the SIVS.
13	doc-13_SIVS_Phase I_ Stat Report_04-11-04.pdf	Statistical report on the results for Phase I of the SIVS involving the EpiSkin, EpiDerm and SIFT test methods. *[See also document Nr. 6 on the statistical evaluation of*

		the Phase II results].
14	doc-14_Hartung_2004_Modular Approach.pdf	This paper describes the modular approach used by ECVAM for purposes of test method validation. The modular approach dissects the information requirements for validation into 7 distinct modules and provides a flexible approach to validation by allowing the information for each module to be furnished at different time points.
15	doc-15_Botham-TaskforceReport-1_1998.pdf	The ECVAM Skin Irritation Task Force Report from 1998 on the status of alternative (including in vitro) approaches to skin irritation testing.
16	doc-16_Faller_TIV_2002.pdf	Scientific publication in which human patch test results are compared to testing results obtained with three reconstructed human epidermis models (EpiSkin, EpiDerm and one in-house model from Cosmital).
17	doc-17_Fentem_2001.pdf	Publication of the ECVAM prevalidation study performed from 1999 - 2000
18	doc-18_Zuang-TaskforceReport-2_2002.pdf	Publication of the follow-up work to the ECVAM prevalidation study
19	doc-19_Kandarova_2005.pdf	Publication of the results of the first EpiDerm optimisation study (conducted in 2004) that lead to the original EpiDerm test method protocol subsequently analysed in the ECVAM SIVS (2003-2007). [see document 20 on the EpiSkin optimistation]
20	doc-20_Cotovio_ 2005.pdf	Publication of the results of the EpiSkin optimisation (conducted in 2004) that lead to the common EpiSkin and EpiDerm protocol subsequently analysed in the ECVAM SIVS (2003-2007).
21	doc-21_Hoffmann_2005.pdf	An analysis of prevalence, variability and regulatory classification of industrial chemicals from the EU New Chemicals Database which was a source of the testing set used during the SIVS
22	doc-22_ESAC_il1a rationale-3-1.pdf	Background document to the Interleukin 1 alpha endpoint.
23	doc-23_ESAC26_statement_ SkinIrritation_20070525_C.pdf	ESAC statement (April 2007) on the scientific validity of the EpiSkin and original EpiDerm test methods upon completion of the ECVAM SIVS
24	doc-24_PR consensus report final.pdf	Peer Review Panel Consensus Report concerning the SIVS
25	doc-25_Subset analysis final 11-06-07.doc	Brief analysis of the predictive capacity of the two EpiSkin and original EpiDerm method with respect to the chemicals derived from the New Chemicals Database.
26	doc-26_Spielmann et al. SIVS ATLA 35_2007-1.pdf	Publication of the Skin Irritation Validation Study (SIVS)
27	doc-27_Eskes FINAL.pdf	Publication of the Chemical Selection Process pertaining to the SIVS
28	doc-28_TG skin irritation-final.doc	Draft OECD test guideline

ANNEXE 2 – ESAC STATEMENT APRIL 2007 (EPISKIN AND ORIGINAL EPIDERM)

STATEMENT ON THE VALIDITY OF *IN-VITRO* TESTS FOR SKIN IRRITATION

At its 26th meeting, held on 26-27th April, 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

After a review of scientific reports and peer reviewed publications on the following range of *in-vitro* tests, which had been subjected to a full validation study:

1. EpiDerm (with MTT reduction and IL-1α release);
2. EPISKIN (with MTT reduction and IL-1α release);

of these, the EPISKIN method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement **(based on the performance of the assay as specified in the annex)** for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances. At the present time, the IL-1α endpoint should be regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint.

At this time, due to its high specificity, the EpiDerm model reliably identifies skin irritants, but negative results may require further testing (e.g. according to the tiered strategy, as described in the OECD TG 404). Improvement of the EpiDerm protocol should be made to increase the level of sensitivity.

This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary submissions made by the Management Team; and the considered view of the Peer Review Panel appointed to oversee the process

Thomas Hartung
Head of Unit
ECVAM
Institute for Health & Consumer Protection
Joint Research Centre
European Commission
Ispra 27 April 2007

1. The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

   This statement was endorsed by the following members of the ESAC:

   Ms Sonja Beken (Belgium)
   Ms Dagmar Jírová (Czech Republic)
   Mr Tõnu Püssa (Estonia)
   Mr Lionel Larue (France)
   Mr Manfred Liebsch (Germany)
   Ms Annalaura Stammati (Italy)
   Mr Jan van der Valk (The Netherlands)
   Mr Constantin Mircioiu (Romania)
   Mr Albert Breier (Slovakia)
   Ms Argelia Castaño (Spain)
   Mr Patric Amcoff (Sweden)
   Mr Jon Richmond (UK)
   Mr Carl Westmoreland (COLIPA)
   Ms Vera Rogiers (ECOPA)
   Ms Nathalie Alépée (EFPIA)
   Mr Robert Combes (ESTIV)
   Mr Hasso Seibert (European Science Foundation)

   The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself.

   Mr Thomas Hartung (ECVAM; chairman)
   Mr Jens Linge (ECVAM; ESAC secretary)
   Ms Elke Anklam (Director of IHCP)
   Ms Susanna Louhimies (DG Environment, Unit C.3)
   Ms Barbara Mentré (DG ENTR)
   Ms Grace Patlewicz (ECB, DG JRC)
   Mr Christian Wimmer (DG Research)
   Mr Hajime Kojima (JACVAM)
   Ms Laurence Musset (OECD)
   Mr Barry Philips (Eurogroup for Animal Welfare)
   Mr William Stokes (NICEATM, USA)

ANNEX

General information on the ECVAM skin irritation validation study

After extensive optimisation and prevalidation activities (see background to the SIVS here below), ECVAM launched a formal validation study on three *in vitro* test systems in 2003. Two of the assays employed reconstituted human epidermis models (EPISKIN, EpiDerm) and one, the skin integrity function test (SIFT) employed *ex vivo* mouse skin. The aim of the study was to replace the regulatory Draize skin irritation test (EU B. 4 method; OECD TG 404) currently performed on albino rabbits by assessing the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of these test systems with a set of 58 coded test chemicals. The goal of the study was to evaluate if the *in vitro* tests would predict *in vivo* classification according to the EU classification system using the risk phrase R38 for skin irritants and no classification for non irritants. In addition, the chemical selection was representative for the three categories [strong (category 2), mild (category 3) and non-irritants (no category)] of the Globally Harmonised classification System (GHS) for permitting a post-hoc evaluation of the results according to GHS. The validation study was conducted according to the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34). Furthermore, to ensure a high quality of the commercially produced human skin models, the facilities of the producers of the human skin models EPISKIN and EpiDerm were evaluated by independent auditors at the beginning of the ECVAM Skin Irritation Validation Study (SIVS).

The study was sponsored by ECVAM, coordinated by a main contractor (ZEBET-BfR, Germany) and managed by a Management Team (MT; see table 1 for the composition of MT).

Table 1. Composition of the Management Team of the SIVS

Chair (Dr Phil Botham)
Co-chair (Dr Julia Fentem)
Sponsor representative (Dr Valérie Zuang, *alternate:* Dr Chantra Eskes)
Independent biostatistician (Dr Sebastian Hoffmann)
Representative of the main contractor (Dr Horst Spielmann)
Representative of the CSSC (Dr Andrew Worth)
ECB customer (Dr Thomas Cole)
Representatives of the test systems:
EPISKIN (Dr Roland Roguet)
EpiDerm (Dr Manfred Liebsch)
SIFT (Dr Jon Heylings)
Observers from the US:
ICCVAM (Dr Karen Hamernik; *alternate*: Dr Abby Jacobs)
NICEATM (Dr William Stokes; *alternate*: Dr Ray Tice)

A Chemicals Selection Sub-Committee (CSSC) was appointed to identify test chemicals to be used in the SIVS having high quality existing *in vivo* data with which to correlate the *in vitro* measurements. Since chemicals from the European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC) database of reference chemicals for skin irritation/skin corrosion had been extensively used in the preceding studies, the CSSC was requested to make use of novel sources for potential test chemicals. For this purpose chemicals were selected from the New Chemicals Database (NCD) which is the central archive within the EU notification scheme for new commercial chemicals. In addition, existing chemicals readily available from major manufacturing and/or distribution sources were selected from alternative databases such as the Toxic Substance Control Act (TSCA) database maintained by the US Environmental Protection Agency (EPA) and the ECETOC database, excluding those chemicals used in the previous optimisation and prevalidation phases.

A total set of 58 chemicals comprising a set of 25 existing chemicals and 33 chemicals from the NCD were selected and tested in the SIVS. The selected chemicals (a) represented statistically justified sample sizes for distinguishing R38 from non classified chemicals, (b) provided a balanced representation of the three GHS categories to allow for post-hoc evaluation of the performance of the assays for that classification system, and (c) accounted as far as possible the large prevalence known to exist for chemicals which have oedema and erythema scores of 0. These chemicals were independently coded and distributed to the participating laboratories. The selected chemicals presented a variety of molecular structures, functional chemical groups, effect and use categories, as well as a wide range of physical-chemical properties. They represented a challenging set of chemicals relevant to current industrial commerce for the alternative methods being validated.

In phase 1 of the ECVAM SIVS, 20 chemicals (9 irritant, 11 non irritant) from NCD were tested under blind conditions in the lead laboratories (EPISKIN - L'Oreal, EpiDerm - ZEBET, SIFT - Syngenta). The methods applied (with Standard Operating Procedures, SOP's) were the refined, optimised protocols developed after the ECVAM prevalidation study. For the human skin model assays this consisted in applying the test chemicals to the surface of the skin for 15 minutes, followed by a post-treatment incubation period of 42 hours, and the subsequent assessment of their effects on cell viability by using the MTT assay.

The prediction model related to MTT used in Phases 1 and 2 was the following:

> "The test substance is considered to be irritating to skin (R38), if the tissue viability after exposure and post incubation is less or equal (≤) to 50%".

When cell viability (MTT reduction) was used as endpoint, the two skin models met the acceptance criteria set by the MT of the study. While the specificity (correct prediction of non-irritants) of both the EPISKIN and EpiDerm assays was 91%, their sensitivity (correct prediction of R38 irritants) was 67% and 56%, respectively. However, since almost all of the misclassified chemicals were lying at the threshold between irritant and non-irritant chemicals according to the EU classifcation scheme, the MT concluded that the predictive capacity of both Epiderm and EPISKIN was sufficient to justify them to proceed to Phase 2. On the other hand, the predictive capacity of the SIFT method was considered inadequate. For SIFT, it was suggested that the lead laboratory re-evaluated the test protocol and prediction model, particularly in relation to the manner in which solids and non-surfactant materials are handled.

In Phase 2, all 58 chemicals were assessed in three different laboratories for each of the two reconstituted human skin methods. The EpiDerm test was conducted in the following laboratories: ZEBET (lead lab), Germany; Institute for *In vitro* Sciences (IIVS), USA; and BASF, Germany. The EPISKIN test was conducted in the following laboratories: L'Oréal (lead lab), France; Unilever, UK; and Sanofi-Synthélabo, France. Chemicals were re-coded from Phase 1 to ensure blind testing. The main endpoint measured for both Epiderm and EPISKIN was cell viability measured by MTT reduction, as used in all previous testing. However, a second endpoint, interleukin-1α release, was added for those chemicals which did not reduce cell viability below the threshold for predicting irritancy, to determine if it could be used to improve the sensitivity of the assays. This second endpoint was used in all three laboratories assessing EPISKIN and by the lead laboratory for Epiderm.

The prediction model related to the combined use of MTT and IL-1α release in Phase 2 was the following:

The test substance is considered to be **irritant** to skin: if the viability after 15 minutes of exposure and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1α release is more (>) than 60pg/ml.
The test substance is considered to be **non irritant** to skin: if the viability after 15 minutes of exposure and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1α release is less or equal (≤) to 60pg/ml.

The predictive capacities of the assays in this second phase are shown in Table 2. The within-laboratory reproducibility of classifications over three independent experiments meeting the acceptance criteria was 93.9% for EPISKIN (MTT) and 96.0% for EpiDerm (MTT). The between-laboratory reproducibility measured as the proportion of identical median classifications between laboratories was 89.5% for EPISKIN (MTT) and 88.5% for EpiDerm.

Table 2. Predictive capacities of EPISKIN and EpiDerm (MTT: based on the median classification per laboratory; MTT+IL1α: based on the classification derived from the mean viability of the independent experiments per chemical and laboratory)

	EPISKIN (MTT)	***EPISKIN (MTT+IL1-α)***	***EpiDerm (MTT)****
Sensitivity	**74.7%**	**90.7%**	57.3%
Specificity	**80.8%**	**78.8%**	83.8%
Concordance/Accuracy	**78.2%**	**83.0%**	72.4%

*The addition of IL-1α to the EpiDerm protocol gave no improvement to the outcome

The study was forwarded to the ECVAM Scientific Advisory Committee (ESAC) with a proposal that EPISKIN could be considered as a replacement for the rabbit skin irritation method and Epiderm as a constituent of a testing strategy.

Background to the ECVAM skin irritation validation study

In 1998, the ECVAM Skin Irritation Task Force published a report on the actual status of *in vitro* skin irritation testing and proposed 10 "challenge chemicals" for which promising, concordant *in vivo* data from the rabbit test, *in vivo* data from 4hr human patch test, and *in vitro* data from the human skin model EpiDerm were available. Proponents of new *in vitro* test systems were encouraged to submit data obtained with new *in vitro* skin irritation test protocols for these chemicals (1) for assessment whether these tests could be considered in an ECVAM prevalidation study. At the same time the suitability of various endpoints for prediction of human skin irritation was evaluated in an EU 4th framework collaborative project in several human reconstructed skin models, revealing cell viability reduction (MTT reduction) and IL-1α release the most promising endpoints. Because MTT reduction and IL-1α release showed a high inter-correlation, and IL-1α release was more variable, MTT-reduction was proposed to be the best endpoint for human skin models (2).

Of the test systems for which data were submitted to the ECVAM TF, five tests [perfused pig-ear, Prediskin, SIFT, EPISKIN, EpiDerm] had been considered promising for participation in the ECVAM prevalidation study. However, during the prevalidation study, two tests failed already in phase 2 due to insufficient reproducibility, whereas the other tests [SIFT, EPISKIN and EpiDerm] showed a sufficient intra- and interlaboratory reproducibility, but failed in their ability to correctly predict the skin irritation potential of 20 chemicals that were tested in phase 3 of the ECVAM prevalidation study (3). The ECVAM Management Team of the study therefore proposed refinement and optimisation of these three tests before considering them for formal validation.

In 2001, the ECVAM Skin Irritation Task Force and the laboratories responsible for the refinement of the tests met again and discussed ways forward to approach formal validation. In addition, since a post hoc analysis of prevalidation data for MTT reduction for EPISKIN and EpiDerm revealed similar sensitivity, it was recommended to develop a common test protocol for both skin models before the start of a formal validation study (4).

In November 2002, the ECVAM Skin Irritation Task Force (TF) discussed the refinements of the SIFT (5) and the skin model tests (6) and came to the conclusion that processing the tests to formal validation could be recommended. However, because all refinements were made using the 20 chemicals from the prevalidation study, the TF recommended to perform the SIVS in two phases: a first phase (phase 1) for the confirmation of the refinements made by the leading labs Syngenta (SIFT), L'ORÉAL (EPISKIN), and ZEBET (EpiDerm) by testing new chemicals in a controlled way under blind conditions. If the outcome of phase 1 were still promising, the tests would proceed to a second phase (phase 2), i.e. in a blind trial involving three laboratories per test.

During 2003, the EPISKIN test was further refined by L'OREAL by extending the post incubation period of the tissues (after 15 min chemical exposure) to 42 hours which allowed significant effects to increase, and recovery from weak effects.

In May 2003, an ECVAM Stakeholder Workshop recommended to conduct a formal validation study and to concentrate on the predictions of the EU classification system (R38 vs. no label), because the tests were developed and optimised for this classification scheme. L'ORÉAL and ZEBET collaborated then in developing a common test protocol to be used in the ECVAM SIVS, and evaluated it first with the 20 "challenge" chemicals of the ECVAM prevalidation study. In 2004, upon request of the ECVAM SIVS Management Team and in parallel to performing phase 1 of the SIVS, the database was further increased by testing all non-corrosive chemicals recommended in the ECETOC reference data base (ECETOC report No. 66). The data obtained in both skin models with the optimised common protocol were very promising, and published back to back in 2005 (7,8). The BfR was contracted in November 2003 further to the publication of a call for tender for the ECVAM SIVS by the European Commission in June 2003. The

study started formally with the 1st Meeting of the SIVS Management Team (MT) on 17-18 November 2003.
Manuscripts on the outcome of the skin irritation validation study and on the chemicals selection, are currently being finalised for publication.

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ANNEXE 3 – ESAC STATEMENT NOVEMBER 2008 (MODIFIED EPIDERM AND SKINETHIC)

STATEMENT ON THE SCIENTIFIC VALIDITY OF IN-VITRO TESTS FOR SKIN IRRITATION TESTING

At its 29th meeting, held on 4-5th November, 2008 at the European Commission in Brussels, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the following statement:

Two *in-vitro* skin irritation tests have been evaluated according to the principles outlined in the ECVAM document 'Performance Standards for Applying Human Skin Models to in-vitro skin irritation testing' (1). These performance standards were used to evaluate the reliability and accuracy of two test methods which are both based on reconstructed human epidermis and which measure or predict the same biological or toxic effect as the fully validated and accepted reference method (see ESAC statement, 2007 and validation study report) (2, 3).

A review of the data submitted on the following studies was conducted by an ESAC peer review panel:

1. EpiDerm SIT – update validation study: modification of the validated EpiDerm Test (MTT endpoint)

2. SkinEthic RHE assay –external catch up validation study (MTT endpoint)

It is concluded that the performance of these assays in these studies met the criteria outlined to be considered to have sufficient accuracy and reliability for prediction of R38 skin irritating and no-label (non-skin irritating) test substances compared to the validated and accepted method. Limitations associated with the previously validated and accepted in-vitro method for skin irritation (e.g. applicability domain) also apply to the two tests reviewed here (Ref. 4).

This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary material made available to the Peer Review Panel by ECVAM; and the considered view of the Peer Review Panel appointed to oversee the process.

Joachim Kreysa
Head of Unit
In-Vitro Toxicology Unit
European Centre for the Validation of Alternative Methods
5th November 2008

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The ESAC was established by the European Commission, and is composed of nominees from the EU Member States, industry, academia and animal welfare organisations, together with representatives of the relevant Commission services.

This statement was endorsed by the following members of the ESAC:

Ms Sonja Beken (Belgium)
Mr Albert Breier (Slovakia)
Ms Maija Dambrova (Latvia)
Ms Katalin Horvath (Hungary)
Ms Dagmar Jírová (Czech Republic)
Mr Roman Kolar (Eurogroup for Animals)
Ms Elisabeth Knudsen (Denmark)
Mr Manfred Liebsch (Germany)
Mr Lionel Larue (France)
Mr Gianni Dal Negro (EFPIA)
Mr Efstathios Nikolaidis (Greece)
Mr Constantin Mircioiu (Romania)
Mr. Walter Pfaller (Austria; moderator)
Mr Jon Richmond (UK)
Ms Vera Rogiers (ECOPA)
Mr Hasso Seibert (ESF)
Mr Dariusz Sladowski (Poland)
Mr Jan van der Valk (The Netherlands)
Mr Carl Westmoreland (COLIPA)
Mr Timo Ylikomi (Finland)

The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself:

Ms Elke Anklam (IHCP; chairman)
Mr Joachim Kreysa (ECVAM)
Mr Jürgen Büsing (DG RTD)
Ms Silvia Casati (ECVAM, DG JRC)
Mr Thomas Cole (ECVAM, DG JRC, ESAC secretary)
Ms Laura Gribaldo (ECVAM, DG JRC)
Mr Claudius Griesinger (ECVAM, DG JRC)
Ms Eimear Kelleher (IHCP)
Ms Karin Kilian (DG SANCO)
Ms Barbara Mentré (DG ENTR)
Ms Pilar Prieto (ECVAM, DG JRC)
Mr Juan Riego Sintes (CPSQ, DG JRC)
Ms Sigrid Weiland
Ms Valérie Zuang (ECVAM, DG JRC)
Mr Patric Amcoff (OECD)
Mr Hajime Kojima (JaCVAM)
Mr William Stokes (NICEATM)
Mr Raymond Tice (NICEATM)
Ms Marilyn Wind (ICCVAM)

Informative Annex

ECVAM BACKGROUND INFORMATION ON THE VALIDATION OF TWO *IN VITRO* TEST METHODS FOR SKIN IRRITATION TESTING PERFORMED ON THE BASIS OF PERFORMANCE STANDARDS

Claudius Griesinger, Ispra, Italy, 11 November 2008

1. Background to Validation Studies based on Performance Standards

The ECVAM Performance Standards for applying human skin models to in vitro skin irritation testing (1) are based on the specifications of the two skin models that were validated during the ECVAM skin irritation validation study (SIVS), the commercially available EPISKIN and the EpiDerm test methods (2-4).

The Performance Standards describe guidance and minimum performance criteria that novel 'me-too' or modified test methods should fulfil so that they may be considered scientifically valid. The performance criteria include *inter alia* (a) a description of general and functional model conditions including acceptance criteria regarding the quality of individual tissues used as test system, (b) test acceptance criteria (e.g. guidance values for positive and negative control), (c) guidance regarding the test procedure and data interpretation (prediction model), (d) 20 reference chemicals that constitute a representative set of chemicals used during the full prospective validation study (3) as well as (e) performance criteria for test method reliability and predictivity.

The Performance Standards are intended as a tool to aid the evaluation, assessment and validation of novel methods on the basis of an experimental testing set of chemicals (the PS reference chemicals) that is markedly reduced in comparison with that of a full prospective validation study. According to OECD guidance document Nr. 34 on the validation and international acceptance of new or updated test methods for hazard assessment (5), two types of test methods can be evaluated on the basis of performance standards. These are

c) Test methods that are sufficiently similar with regard to structural and functional parameters in comparison with the validated methodology ("similar methods" or "me-too methods"). The corresponding validation process is referred to as "catch-up validation".

d) Modifications of validated methods ("modified methods") which are minor enough to warrant the limited experimental assessment as outlined in the Performance Standards. The corresponding validation process is referred to as "update validation".

2. Validation of two *in vitro* skin irritation methods in reference to the ECVAM in vitro Skin Irritation Performance Standard

2.1 Test methods endorsed

The two test methods endorsed by the 29th ESAC are

a) The SkinEthic RHE model, a similar/me-too method, submitted to ECVAM as a non-ECVAM coordinated catch-up study. The test was confirmed by ECVAM as sufficiently similar with regard to its structural and functional characteristics in reference to the Performance Standards and the test method was therefore admitted as a *non-ECVAM coordinated catch-up validation study*.

b) The EpiDerm SIT model, a modification of the previously validated EpiDerm method (2), submitted to ECVAM as a non-ECAVM coordinated update validation study. The main modification performed is the prolongation of the exposure time to the test substances from 15 ('common protocol', ECAVM SIVS) to 60 minutes, while all other essential model parameters remained unchanged. The test method was therefore admitted by ECVAM as a *Non-ECVAM coordinated update validation study*.

It is important to note that all human reconstructed tissue models that have been validated so far for the assessment of skin irritancy potential of xenobiotics, use a postincubation time of 42 hours. However, the assays differ with regard to the exposure time employed, i.e. the period that the epidermal surface is acutely treated with the xenobiotic. In contrast to the relatively short exposure time of 15 minutes outlined in the so-called "common protocol" of the ECVAM SIVS (3), the assays validated in the current context use extended exposure times: the modified EpiDerm SIT assay features, as stated above, an exposure time of 60 minutes while the SkinEthic RHE uses an exposure time of 42 minutes. The exposure times are understood to reflect the different barrier properties of the test systems and are adjusted for each test system in order to guarantee a dynamic response: the exposure time needs to be long enough to allow the development of measurable effects while being short enough to ensure that the system is not driven into saturation.

2.2 Submission, evaluation and peer review process

The SkinEthic RHE test method had been submitted by SkinEthic Laboratories, Nice, France on 7 April 2008. The EpiDerm SIT test method had been submitted on 23 April 2008 by the Federal Institute for Risk Assessment (BfR), Berlin, Germany.

Both test method submissions were evaluated by ECVAM on the basis of the criteria laid out in the ECVAM performance standards document (1). In addition to the external assessment of transferability provided in both test method submissions, the transferability of the SkinEthic RHE method as well as its standard operating procedure (SOP) were independently assessed and confirmed in-house at ECVAM from March to May 2008. Such independent assessment by ECVAM was deemed not necessary in the case of the EpiDerm SIT method since the EpiDerm model had undergone extensive assessment during the full skin irritation validation study (2-4) and since the modification of the test method was considered minor.

After ECVAM evaluation, the test method submissions and additional auxiliary material made available by ECAVM were reviewed by an ESAC Peer Review Panel and independently evaluated by this panel with regard to the ECAVM Performance Standards (1). The Peer Review Process was finalised on September 8, 2008.

3. Endpoints assessed by the two test methods

Both tests use the MTT test as primary endpoint. This colorimetric assay for cell viability is based on the mitochondrial reduction of the vital dye MTT [3-(4,5-Dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide] to a purple-coloured formazan. Cell viability has been demonstrated to be a suitable parameter to extrapolate on the irritancy potential of chemicals in human reconstructed epidermis models (3,4).

In addition, both the SkinEthic RHE and EpiDerm submission provided information on the secondary endpoint IL-1α (Interleukin 1 alpha). The data on IL-1α submitted in both dossiers did not demonstrate an improvement of the predictive capacity of the test methods. Therefore, for both methods, only the data for the MTT endpoint were considered with regard to the predictive capacity.

Background to the IL1 α endpoint:

As a result of the ECVAM SIVS, the IL-1α endpoint had been suggested as a potentially useful adjunct (2). IL-1α is an inflammatory mediator secreted by the non-classical pathway (6,7). The ECVAM SIVS had concluded that IL 1α may be capable, under certain conditions, to increase the sensitivity of human reconstructed epidermis assays (2-4), e.g. when used in a tiered testing approach to identify false negatives of the MTT endpoint.

4. Predictive values of the two test methods

Considering the MTT endpoint, the two validated method have predictive values as shown in Table 1, calculated on the basis of the *median (or mode) of the individual laboratory predictions* for each of the 20 reference chemicals. For comparison, the corresponding values for the reference method EPISKIN are provided. Both submitted test methods meet the values of predictivity indicated in the performance standards (specificity = 80% and sensitivity = 70%).

Table 1: *Predictive values (in %) for the MTT endpoint of the two novel validated in vitro tests for skin irritation testing (SkinEthic RHE and modified EpiDerm SIT) in comparison to the fully validated reference method (EPISKIN) of the ECVAM skin irritation validation study.*

	EPISKIN (reference method)	**Modified EpiDerm SIT**	**SkinEthic RHE**
Specificity	80	**80**	**80**
Sensitivity	70	**80**	**90**
False positive rate	*20*	***20***	***20***
False negative rate	*30*	***20***	***10***
Accuracy	75	**80**	**85**

5. References

1. ECVAM (2007) *Performance Standards for Applying Human Skin Models to In Vitro Skin Irritation Testing.* Online: http://ecvam.jrc.it/
2. ECVAM (2007) *Statement of the ECVAM Scientific Advisory Committee (ESAC) on the Validity of In Vitro Tests for Skin Irritation.* Online: http://ecvam.jrc.it/
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4. Spielmann, H., mailto:Hoffmann, S., Liebsch, M., Botham, P., Fentem, J., Eskes, C., Roguet, R., Cotović, J., Cole, T., Worth, A., Heylings, J., Jones, P., Robles, C., Kandárová, H., mailto:Gamer, A., Remmele, M., Curren, R., Raabe, H., Cockshott, A., Gerner, I. and Zuang, V. (2007) *The ECVAM International Validation Study on In Vitro Tests for Acute Skin Irritation: Report on the Validity of the EPISKIN and EpiDerm Assays and on the Skin Integrity Function Test.* ATLA **35,** 559-601.
5. OECD (2005) *OECD guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD series on testing and assessment Nr. 34.*
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ANNEXE 4 – ESAC STATEMENT APRIL 2009 (PERFORMANCE OF THE THREE VALIDATED METHODS UNDER UN GHS)

STATEMENT ON

THE PERFORMANCE UNDER UN GHS OF THREE IN-VITRO ASSAYS FOR SKIN IRRITATION TESTING

AND

THE ADAPTATION OF THE REFERENCE CHEMICALS AND DEFINED ACCURACY VALUES OF THE ECVAM SKIN IRRITATION PERFORMANCE STANDARDS

At its 30th meeting, held on 9 and 10 March 2009, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC) unanimously endorsed the following statement, subject to editorial finalisation by the ESAC secretariat and final ESAC consensus established by written procedure as of **9th April 2009**:

1. Performance of the ECVAM-validated skin irritation in vitro tests under UN GHS

Previously, three reconstructed human epidermis models (the EpiSkin, the modified EpiDerm SIT and the SkinEthic RHE test methods) have been validated by ECVAM primarily according to the previous EU classification system which is being replaced over the next years by the new classification system of the CLP regulation (see below) which is based on the United Nations' *Globally Harmonised System of Classification and Labelling of Chemicals* (GHS; Ref. 1). For classification according to the new CLP rules the following deadlines apply: 1 December 2010 for the classification of substances and 1 June 2015 for the classification of mixtures (i.e. preparations). Importantly, the selection of test substances used for the ECVAM skin irritation validation study (SIVS; Ref. 2), performed from 2003 to 2007, already took account of the upcoming UN GHS classification system. Upon completion of the ECVAM SIVS, the EpiSkin test method was found to be a reliable stand-alone method for distinguishing between skin irritants and non-irritants (ESAC statement from April 2007, Ref. 3) and, hence, its performance as reference method with regard to the predictive values was used for specifying the ECVAM skin irritation Performance Standards in May 2007. The modified EpiDerm SIT and the SkinEthic RHE test methods were subsequently validated on the basis of these Performance Standards using the 20 defined Reference Chemicals (ESAC statement from November 2008, Ref. 4).

In December 2008, the EU adopted the UN Globally Harmonised System (UN GHS) for Classification and Labelling and will implement this by means of the Regulation on the Classification, Labelling and Packaging of Substances and Mixtures (CLP Regulation EC 1272/2008; Ref. 5) which came into force on 20 January 2009 and will, after a transitional period, replace the previous EU legislations for the classification of substances and mixtures (i.e. preparations). In agreement with the provisions of the

UN GHS system, the new CLP skin irritation classification system will use a single irritant category (category 2) and hence continues to use a total of two classification categories to distinguish irritant (category 2) from non-irritant (no-category) substances. However, according to the GHS rules for skin irritation classification and labelling, the cut-off score to distinguish between no-category and category 2 substances was shifted to an *in vivo* score of greater or equal 2.3 from a value of 2.0 (previous EU classification system). Consequently substances with an *in vivo* score between 2.0 and 2.3 that are considered irritant under the previous EU classification system will be considered non-irritants under the future CLP classification system, which does not implement the optional additional UN GHS category 3 ("mild irritants": substances with scores greater or equal to 1.5 and smaller than 2.3), which is available for those authorities (e.g. pesticides) that want to have more than one skin irritant category (Ref. 1).

The performance of all three tests under CLP (i.e. UN GHS using one single irritant category) has now been re-evaluated taking this shift of the cut-off value into consideration and has been found satisfactory (Table 1). While the specificity of the EpiSkin method is decreased from 81.8%* (previous EU system) to 71.1 %* (CLP), the test sensitivity has increased from 72%* (previous EU system) to 84.6%* (CLP). The two other methods show similar values for the specificity (both tests 69.2%*), and higher sensitivity values than the reference method under CLP.

The original ESAC statements relating to the scientific validity of these test methods therefore continue to be accurate and, with regard to their use in the context of decisions of classification, can now be extended to the CLP system. Updated accuracy values under CLP are provided in this statement.

Moreover, on the basis of the documentation available confirming the overall satisfactory performance of the three methods, the ESAC is of the opinion that no further work is required at this stage and that the existing information on the validation studies and additionally available background information is sufficient to explain and justify the changes in performance of the tests and key aspects of the performance standards (i.e. reference chemicals and defined accuracy values) necessitated by the threshold shift upon adaptation of the GHS system in the EU. As is common practice, adaptations to technical progress should be performed as appropriate and necessary. It should be noted, that any conclusions on the applicability domain are based, at this stage, mainly on the testing set used during the ECVAM SIVS.

*) All values are based on the final predictive decisions of the study calculated on the basis of the median of the individual laboratory predictions. Since the predictions are essentially categories (i.e. positive or negative) and take values of either 1 or 0, the final decision can be derived by using either the median or the mode.

Table 1. Accuracy values for the three ECVAM-validated skin irritation in vitro test methods under CLP (UN GHS)

	EpiSkin test method (58 chemicals[1])	EpiSkin test method (20 reference chemicals[3])	Modified EpiDerm test method (20 reference chemicals[3])	SkinEthic test method (20 reference chemicals[3])
Specificity (%) [2]	**71.1**	76.9	69.2	69.2
Sensitivity (%) [2]	**84.6**	85.7	85.7	100
Overall Accuracy (%) [2]	**74.1**	80	75	80

[1]) The test substances from the ECVAM Skin Irritation Validation Study (SIVS) conducted from 2003 to 2007.

[2]) Based on the median (or mode) of the individual laboratory predictions.

[3]) Original 20 RC from the ECVAM Performance Standards May 2007

2. Adaptation of the Reference Chemicals and Defined Accuracy Values of the ECVAM Performance Standards

2.1 Updated list of Reference Chemicals

Due to the threshold shift resulting from the adoption of the UN GHS system in the EU, the reference chemicals of the original ECVAM Performance Standards were no longer properly balanced with regard to an equal representation of Irritant versus Non-irritant substances.

To address this and other issues (i.e. global commercial availability, evidence that some substances are non-irritant in human, handling qualities) the reference chemical set was updated. The updated reference chemical set reflects the false negative and false positive rates obtained with the EpiSkin method under UN GHS on the basis of the full set of 58 test substances from the ECVAM skin irritation validation study allowing for the appropriate future validation of modified or similar ("me-too") test methods.

Deletions

The following six substances were deleted (in vivo scores in parentheses):

1) d-propylene glycol (0)
2) allyl heptanoate (1.7)
3) terpinyl acetate (2.0)
4) tri-isobutyl phosphate (2.0)
5) alpha-terpineol (2.7)
6) butyl methacrylate (3.0)

Additions

The following six substances were added (in vivo scores in parentheses):

1) cinnamaldehyde (2.0)
2) 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl (2.7)
3) 5% potassium hydroxide (3.0)
4) benzenethiol, 5-(1,1-dimethyl)-2 methyl (3.3)
5) 1-methyl-3-phenyl-1-piperazine (3.3)
6) 1,1,1-trichloroethane (4.0)

Moreover, the updated reference chemicals (table 2) meet the following criteria:

1. the chemicals are commercially available
2. they are representative of the full range of Draize skin irritancy scores (from non-irritant to strong irritant)
3. they have a well-defined chemical structure
4. they are representative of the chemical functionalities used in the validation process
5. they are not associated with an extremely toxic profile (e.g. carcinogenic or toxic to the reproductive system) and they are not associated with prohibitive disposal costs.

Table 2: Updated reference chemicals

Nr.	Reference Chemical	*In vivo* Score	EU *in vivo* category	GHS-EU *in vivo* category	EPISKIN classifi-cation
1	1-bromo-4-chlorobutane	0	no	no category	**I**
2	diethyl phthalate	0	no	no category	NI
3	naphthalene acetic acid	0	no	no category	NI
4	allyl phenoxy-acetate	0.3	no	no category	NI
5	isopropanol	0.3	no	no category	NI
6	4-methyl-thio-benzaldehyde	1	no	no category	**I**
7	methyl stearate	1	no	no category	NI
8	heptyl butyrate	1.7	no	optional cat. 3	NI
9	hexyl salicylate	2	R38	optional cat. 3	NI
10	cinnamaldehyde	2	R38	optional cat. 3	**I**
11	1-decanol *	2.3	R38	category 2	I
12	cyclamen aldehyde	2.3	R38	category 2	I
13	1-bromohexane	2.7	R38	category 2	I
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	2.7	R38	category 2	I
15	5% potassium hydroxide	3	R38	category 2	I
16	di-n-propyl disulphide *	3	R38	category 2	**NI**
17	benzenethiol, 5-(1,1-dimethylethyl)-2-methyl	3.3	R38	category 2	I
18	1-methyl-3-phenyl-1-piperazine	3.3	R38	category 2	I
19	heptanal	3.4	R38	category 2	I
20	1,1,1- trichloroethane	4	R38	category 2	I

*) Substances which are irritant in the rabbit but for which there is reliable evidence that they are non-irritant in humans.

2.2 Updated defined accuracy values as specified in the ECVAM skin irritation Performance Standards

The defined accuracy values (to be included in the ECVAM skin irritation Performance Standards) are derived from the performance of the validated reference method EpiSkin with the updated reference chemicals and under GHS-EU and on the basis of additional considerations relating to relevance in the species of interest. The values are given in table 3.

Table 3: Defined Accuracy Values

	Defined Accuracy Values
Specificity (%)	**70**
Sensitivity (%)	**80**
Overall Accuracy (%)	**75**

Joachim Kreysa
Head of Unit
In-Vitro Methods Unit
European Centre for the Validation of Alternative Methods

Ispra, 9th April 2009

REFERENCES

1. United Nations Economic Commission for Europe (UNECE) (2008). UN Globally Harmonised System of Classification and Labelling of Chemicals, 2nd revised edition; Online: http://www.unece.org/trans/danger/publi/ghs/ghs_rev02/02files_e.html; (as of April 2009).

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3. ECVAM (2007) Statement of the ECVAM Scientific Advisory Committee (ESAC) on the Validity of In Vitro Tests for Skin Irritation. Online: http://ecvam.jrc.it/

4. ECVAM (2008) Statement of the ECVAM Scientific Advisory Committee (ESAC) on the scientific validity of in vitro tests for skin irritation testing. Online: http://ecvam.jrc.it/

5. REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006)

The ESAC was established by the European Commission, and is composed of nominees from the EU Member States, industry, academia and animal welfare organisations, together with representatives of the relevant Commission services.

This statement was endorsed by the following members of the ESAC:

Ms Argelia Castaño(Spain)
Ms Maija Dambrova (Latvia)
Ms Alison Gray (ESTIV)
Ms Katalin Horvath (Hungary)
Ms Maggy Jennings (Eurogroup for Animals)
Ms Dagmar Jírová (Czech Republic)
Mr Roman Kolar (Eurogroup for Animals)
Ms Elisabeth Knudsen (Denmark)
Mr Manfred Liebsch (Germany)
Mr Gianni Dal Negro (EFPIA)
Mr. Walter Pfaller (Austria)
Mr Tõnu Püssa (Estonia)
Mr Jon Richmond (UK)
Ms Vera Rogiers (ECOPA)
Mr Hasso Seibert (ESF, acting as co-moderator at the meeting)
Ms Annalaura Stammati (Italy)
Mr Jan van der Valk (The Netherlands)
Mr Carl Westmoreland (COLIPA, acting as moderator at the meeting)

The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself:

Commission services
Mr Joachim Kreysa (DG JRC, Head of In vitro methods Unit/ECVAM, chairman)
Mr Claudius Griesinger (DG JRC, ESAC secretariat)
Ms Eimear Kelleher (DG JRC)
Ms Karin Kilian (DG SANCO)
Mr Juan Riego Sintes (DG JRC)

The following observers were present
Mr Patric Amcoff (OECD)
Mr Hajime Kojima (JaCVAM)
Mr William Stokes (NICEATM)
Ms Marilyn Wind (ICCVAM)

ANNEXE 5 – ECVAM/CORRELATE TESTING DATA OF THE SKINETHIC TEST METHOD

Test Phase Summary

Date: 06.06.2008
Version: Final
Test Facility Management: Claudius Griesinger. Study Director: Andre Kleensang. Senior Scientist: Siegfried Morath. Testing: Karin Gmeiner.

1. RHE_01 transfer experiment week 22

Testing performed:week 22
By: Karin Gmeiner

1.1 Test items

The test items were 20 non-coded chemicals provided for the RHE_01 transfer experiment from VitoScreen Srl. The set 2B (vials with yellow label caps) was used for this experiment.

1.2 Assay acceptance criteria

Assay acceptance criteria according to SOP "SIVS_RHE_SOP 3.0" chapter 5.8.3, pages 24 - 25 and planned variation 2 (see 1.2):

Negative control
Mean OD value >= 1.2 and SD of viability <= 18% **yes**
Mean OD value = 2.155
SD of viability = 1.1%

Positive control
Mean viability expressed in % of the negative control <= 40% and SD is <=18% **yes**
Mean viability = 0.86%
SD of viability = 0.09%

Transport control PBS
Mean OD value >= 1.2 and SD of viability <= 18% **yes**
Mean OD value = 2.32
SD of viability = 2.31%

Test substance data acceptance criteria **1 of 20 tests failed**
SD value of the calculated three means < 18%

For allyl phenoxy-acetate (CAS Nr. 7493-74-5) the calculated SD from the three mean values was 20.62%.

1.3 Planned variation with regard to the SOP

Variation 1: To assess a possible damage of the RHE skin tissues during the transport in each run another three tissues were treated with PBS (transport control PBS) but without 42 hours post treatment incubation (SOP "SIVS_RHE_SOP 3.0", chapter 5.6, page 18). The assay acceptance criteria for the transport control PBS are given in the SOP "SIVS_RHE_SOP 3.0", chapter 5.8.3, page 25.

Variation 2: In intentional variation to the SOP "SIVS_RHE_SOP 3.0", page 29 the balance verification was performed by an automatic internal calibration as recommended by the manufacturer.

1.4 Deviations with regard to the SOP

No deviations with regard to the SOP.

1.5 Remarks

No remarks

1.6 Results

1.6.1 Test for non-specific reduction of MTT by the test substances

4-methyl-thiobenzaldehyd (CAS Nr.3446-89-7) and cyclamene aldehyde (CAS Nr. 103-95-7) showed a reduction of MTT (SOP "SIVS_RHE_SOP 3.0", chapter 5.2.2, page 11).

1.7 References

1 Hoffmann S (2006) Skin Irritation Validation Study. Phase II: Analysis of the primary endpoint MTT and the secondary endpoint IL-1-α. Version 6 (14 September 2006)

Skin irritation test results

a) Results

Table 1: Test results RHE_01 Transfer Experiment week 22 and comparison to Episkin validation study.

Val Code Nr.	Reference Chemical Name	CAS Number	EU label R38	in vitro Test result	in vitro Test result (descriptive)	Mean viability [%]	SD viability [%]	Comments	Comp. to Episkin validation study			final result EPISKIN
									Lab A	Lab B	Lab C	
C1	1-bromo-4-chlorobutane	6940-78-9	No	**1**	**Positive**	0.95	0.02	**FP**	**FP**	**FP**	**FP**	**FP**
C2	diethyl phthalate	84-66-2	No	0	Negative	100.83	13.84		TN	TN	TN	TN
C3	di-propylene glycol	25265-71-8	No	0	Negative	104.89	5.86		TN	TN	TN	TN
C19	naphtalene acetic acid	86-87-3	No	0	Negative	99.86	4.92		TN	TN	TN	TN
C4	allyl phenoxy-acetate	7493-74-5	No	0	**Failed**	69.21	20.62	SD >18%	TN	TN	TN	TN
C5	isopropanol	67-63-0	No	0	Negative	106.52	4.98		TN	TN	TN	TN
C6	4-methyl-thio benzaldehyd	3446-89-7	No	**1**	**Positive**	2.72	2.60	**FP**	TN	**FP**	**FP**	**FP**
C20	methyl stearate	112-61-8	No	0	Negative	104.63	2.61		TN	TN	TN	TN
C7	allyl heptanoate	142-19-8	No	0	Negative	71.82	14.66		TN	TN	TN	TN
C8	heptyl butyrate	5870-93-9	No	0	Negative	99.10	10.91		TN	TN	TN	TN
C9	hexyl salicylate	6259-76-3	Yes*	**0**	Negative	93.99	5.95	**FN**	**FN**	**FN**	**FN**	**FN**
C10	terpinyl acetate	80-26-2	Yes	1	Positive	2.84	1.54		**FN**	TP	**FN**	**FN**
C11	tri-isobutyl phosphate	126-71-6	Yes	1	Positive	0.91	0.07		TP	TP	TP	TP
C12	1-decanol	112-30-1	Yes	1	Positive	1.25	0.05		TP	TP	TP	TP
C13	cyclamene aldehyde	103-95-7	Yes	1	Positive	1.36	0.04		TP	TP	TP	TP
C14	1-bromohexane	111-25-1	Yes	1	Positive	1.21	0.07		TP	TP	TP	TP
C15	a-terpineol	98-55-5	Yes	1	Positive	0.72	0.11		TP	TP	TP	TP
C16	di-n-propyl disulphide	629-19-6	Yes	1	Positive	0.92	0.01		**FN**	TP	**FN**	**FN**
C17	butyl methacrylate	97-88-1	Yes	1	Positive	0.77	0.08		TP	TP	TP	TP
C18	heptanal	111-71-7	Yes	1	Positive	1.05	0.02		TP	Not tested	Not tested	TP

) negative in humans

b) Predictive values

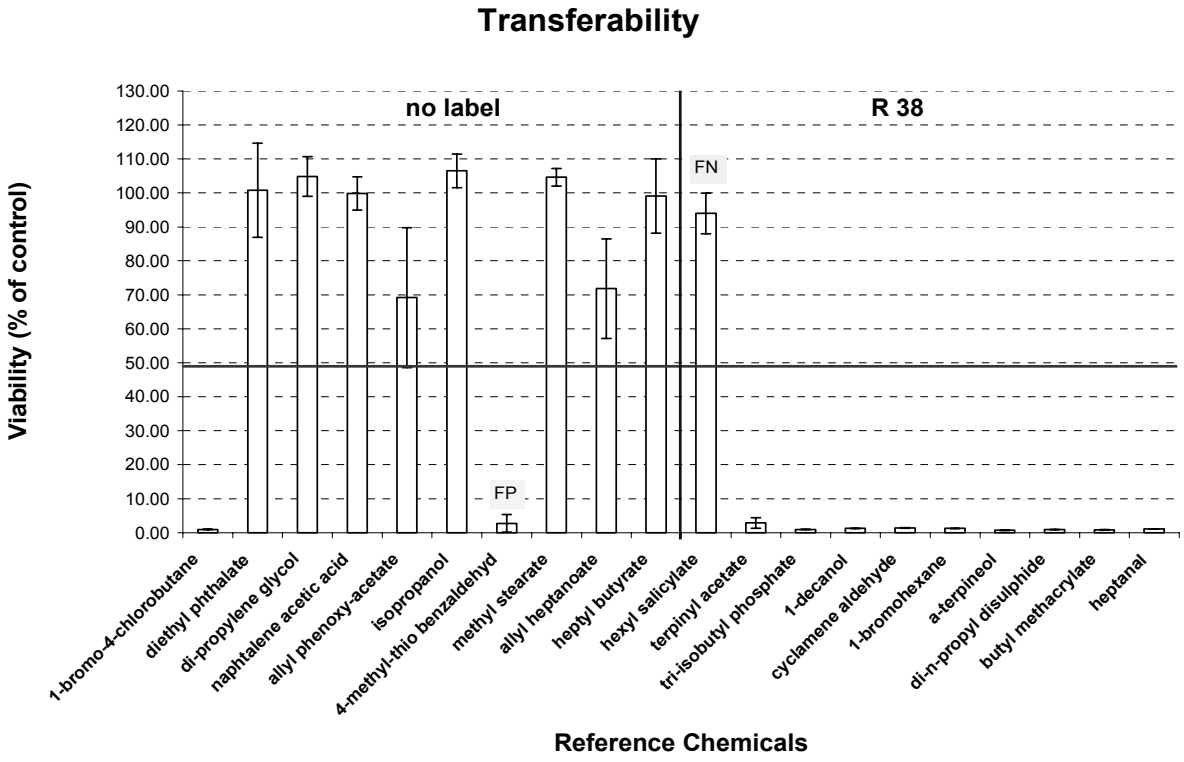
Based on 19 of 20 test items:
Sensitivity = 90%
Specitivity = 77.8%
Predictivity in this set of 20 test chemicals = 84.2% (16 of 19 correct classified, two experiments failed)

Table 2: Predictive values of the transfer experiment

Predictive Values (Correlate Laboratory, 1 run, week 22)

Specificity = TN / (TN + FP)			
Specificity	**77.8**		
Sensitivity = TP / (TP + FN)			
Sensitivity	**90.0**		
		n	
TN	7		
FP	2	9	(failed test run not taken into account)
TP	9		
FN	1	10	
Accuracy = (TP + TN) / (TP+TN+FN+FP)			
Accuracy	**84.2**		

Figure 1: Mean viability [%] and SD of RHE_01 Transfer Experiment week 22. Failed experiment for test items allyl phenoxy-acetate (CAS Nr. 7493-74-5) was not excluded from this figure.



ANNEXE 6 – ECVAM PERFORMANCE STANDARDS FOR SKIN IRRITATION TESTING (UPDATED VERSION, MAY 2009)

Attached as separate document Filename: **PS_invitro_Skin_Irritation_0905-26.pdf**

ANNEXE 7 – RECOMMENDATIONS OF THE 2ND OECD EXPERT CONSULTATION MEETING (ECM), WASHINGTON 15-17 JUNE 2009

► Recommendation 1 the 2nd OECD Expert Consultation Meeting (ECM) (Washington, 15-17 June 2009) on the applicability of the RhE test methods :

The RhE in vitro methods are empirical testing methods and directly address the initial step of the inflammatory cascade/mechanism of action (cell damage and tissue damage resulting in localized trauma). Therefore, there is no scientific reason to assume that these methodologies are not applicable to all substances and mixtures, unless there is specific information that provides evidence regarding such limitations.

►Recommendation Nr. 2 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, 15-17 June 2009) on GHS classification and test method performances:

The OECD ECM is of the opinion that the in vivo classifications used for the recalculation with respect to the UN GHS classification system are correct and consequently the stated test method performances are adequate.

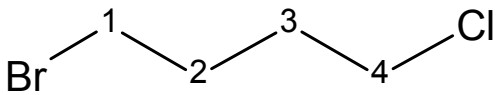
►Recommendation Nr. 3 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, 15-17 June 2009) on the Reference Chemicals (RC) selection:

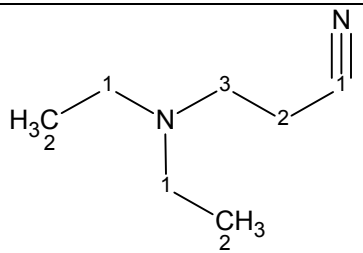
Acknowledging the low prevalence of suitable irritant chemicals and other applicable restrictions that result in a low number of eligible candidate RC, the OECD ECM recommends and endorses the proposed new RC which take up the changes necessitated by the UN GHS system since sufficient supporting testing information is available to substantiate the appropriateness of this RC set for purposes of Performance Standards-based validations and since the proposed RC set is in agreement with the provisions of the OECD Guidance Document Nr. 34 on the validation of testing methods.

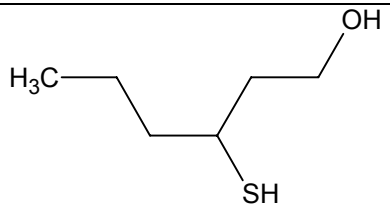
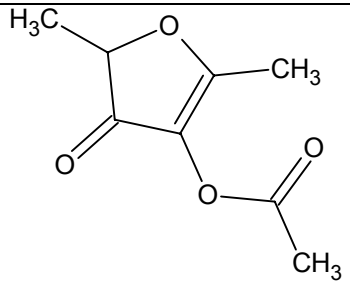
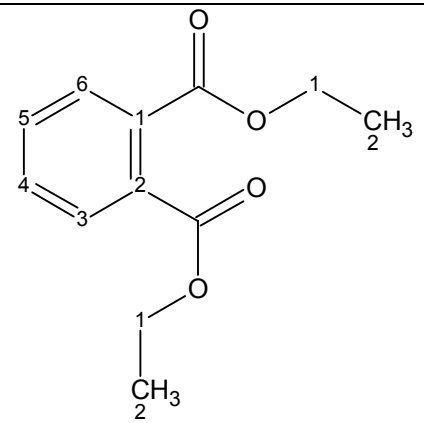
►Recommendation 4 of the 2nd OECD Expert Consultation Meeting (ECM) (Washington, June 15-17) on the revision of OECD Test Guidelines (TG) 404, 430, 431 and 435:

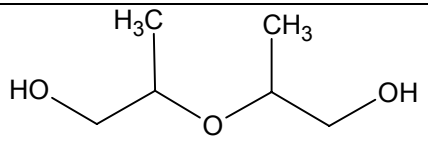
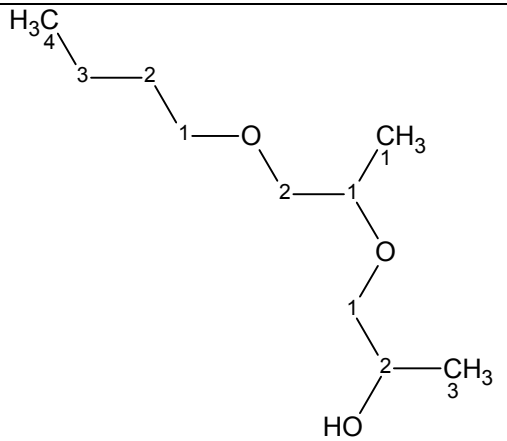
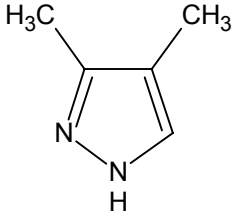
Pending the adoption of the draft OECD TG on *in vitro* skin irritation, the OECD ECM recommends a review and possible update of the OECD TG 404 on skin irritation/corrosion and TG 430, 431 and 435 on skin corrosion.

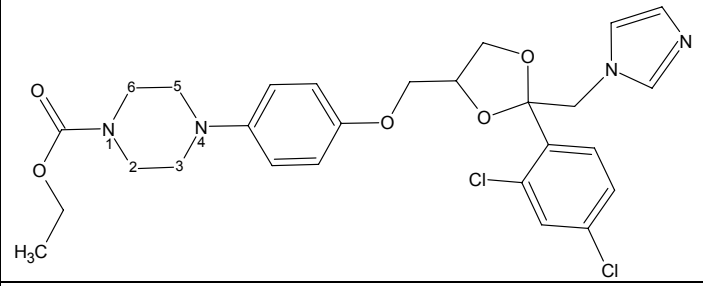
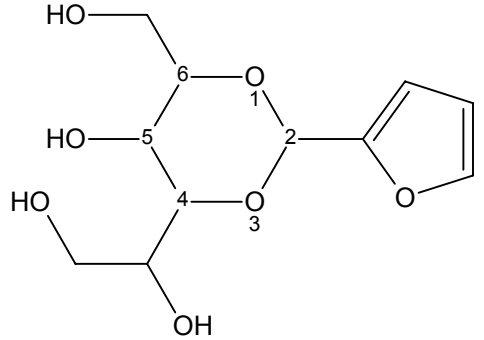
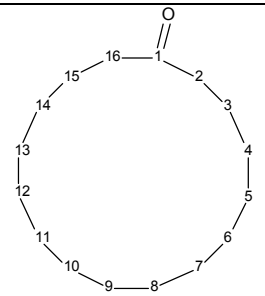
ANNEXE 8 – CHEMICAL STRUCTURES OF THE TEST SUBSTANCES USED DURING THE SIVS

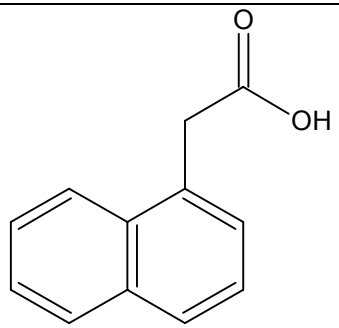
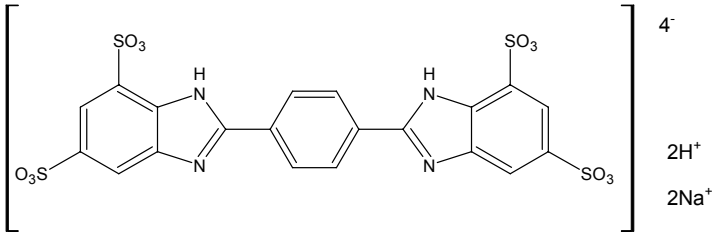
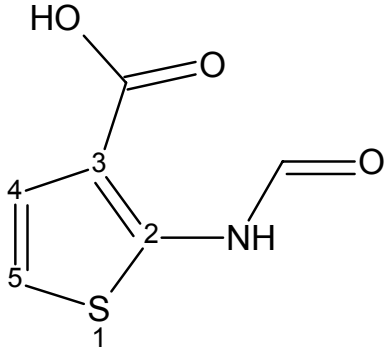
EU/GHS descriptive irritancy	**EC/CAS number** **NCD file leader notification number** **Physical State**	***Substance name (IUPAC from NCD, Annex I Directive 67/548/EEC, ELINCS, EINECS, CAS)*** **Synonym (cited in SIVS)**	**Molecular Structure**	**Classification & Labelling Either: Official EU assignment (published in Annex I, Directive 67/548/EEC)** **Or: NCD Competent Authority assignment (pending official approval and publication)**
1 EU Non-Irritant GHS Non-Irritant	**EC#** **230-089-3** **CAS#** **6940-78-9** Liquid	**<u>EINECS name</u>** 1-bromo-4-chlorobutane		

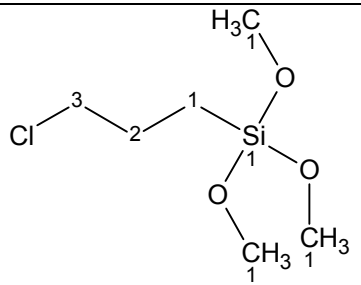
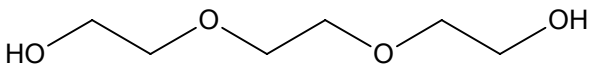
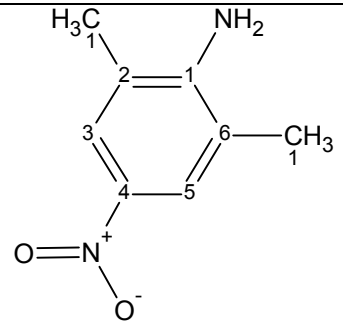
2 EU Non-Irritant GHS Non- Irritant	**EC#** **226-323-9** **CAS#** **5351-04-2** Liquid	**<u>EINECS name</u>** 3-diethylaminopropionitrile		

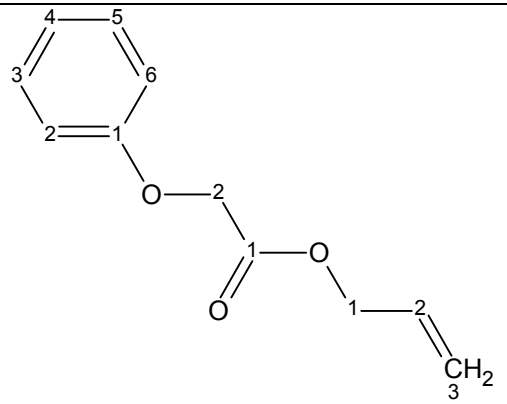
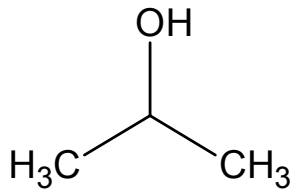
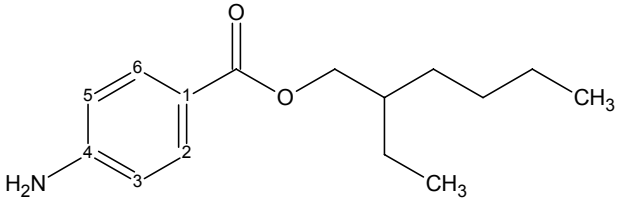
3 EU Non-Irritant GHS Non- Irritant	**EC#** **440-730-0** **CAS#** **51755-83-0** 01-04-1345 Liquid	**IUPAC name (NCD)** 3-mercaptohexanol		Xn; R22 N; R51/53
4 EU Non-Irritant GHS Non- Irritant	**EC#** **435-910-0** **CAS#** **4166-20-5** 01-04-1367 Liquid	**IUPAC name (NCD)** 2,5-dimethyl-4-oxo-4,5-dihydrofuran-3-yl acetate		Xi; R43 N; R51/53
5 EU Non-Irritant GHS Non- Irritant	**EC#** **201-550-6** **CAS#** **84-66-2** Liquid	**EINECS name** diethyl phthalate		

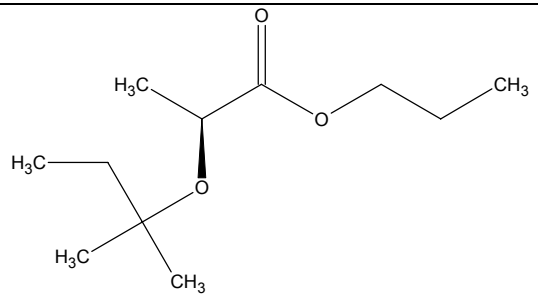
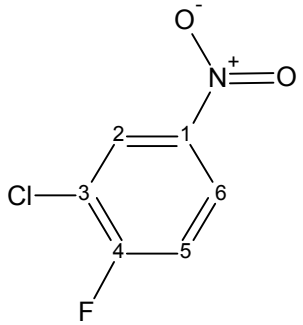
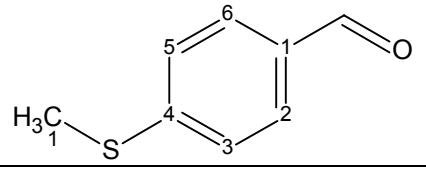
6 EU Non-Irritant GHS Non- Irritant	**EC#** **246-770-3** **CAS#** **25265-71-8** Liquid	**EINECS name** oxydipropanol **Synonym (SIVS)** dipropylene glycol		
7 EU Non-Irritant GHS Non- Irritant	**EC#** **249-951-5** **CAS#** **29911-28-2** Liquid	**EINECS name** 1-(2-butoxy-1-methylethoxy)propan-2-ol **Synonym (SIVS)** dipropylene glycol monobutyl ether		
8 EU Non-Irritant GHS Non- Irritant	**EC#** **429-130-1** **CAS#** **2820-37-3** 98-04-1086 Solid	**IUPAC name (NCD)** 3,4-dimethyl-1*H*-pyrazole		Xn; R22, R41 no symbol; R52, R53

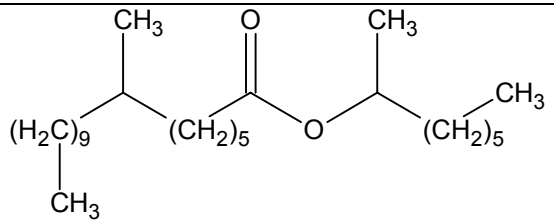
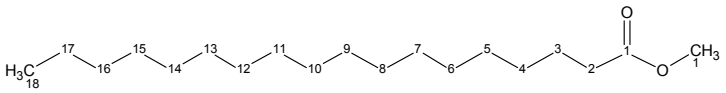
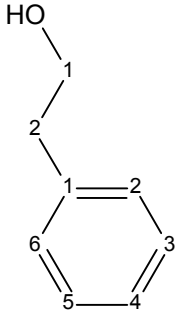
9 EU Non-Irritant GHS Non- Irritant	**EC#** **428-030-3** **CAS#** **67914-69-6** 98-02-0211 Solid	**IUPAC name (NCD)** ethyl cis-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]-methoxy]phenyl]piperazine-1-carboxylate		Xn; R22, R48/22 N; R50/53
10 EU Non-Irritant GHS Non- Irritant	**EC#** **422-440-6** **CAS#** **7089-59-0** 97-05-0281 Solid	**IUPAC name** 2S-(2-furyl)-5R-hydroxy-4R-(1R,2-dihydroxy)ethyl-6S-hydroxymethyl-1,3-dioxane		no classification
11 EU Non-Irritant GHS Non- Irritant	**EC#** **438-930-8** **CAS#** **2550-52-9** 01-04-1394 Solid	**IUPAC name (NCD)** cyclohexadecanone		no symbol; R53

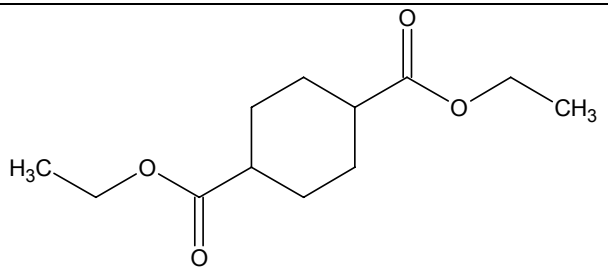
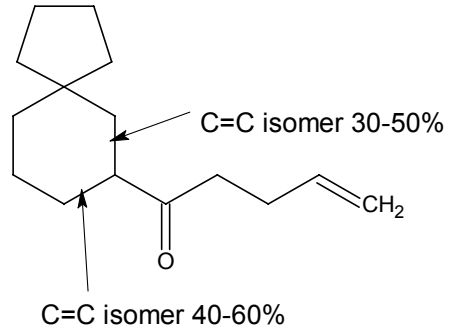
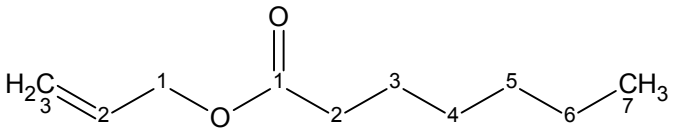
12 EU Non-Irritant GHS Non- Irritant	**EC#** **201-705-8** **CAS#** **86-87-3** Solid	**EINECS name** 1-naphthylacetic acid **Synonym (SIVS)** naphthalene acetic acid		
13 EU Non-Irritant GHS Non- Irritant	**EC#** **429-750-0** **CAS#** **180898-37-7** 98-04-1056 Solid	**IUPAC name (NCD)** disodium 2,2'-(1,4-phenylene)bis-(1H-benzimidazole-4,6-disul fonic acid or monosulfonic acid, monosulfonate or disulfonate		no classification
14 EU Non-Irritant GHS Non- Irritant	**EC#** **431-930-9** **CAS#** **43028-69-9** 99-04-1214 Solid	**IUPAC name (NCD)** 2-(formylamino)-3-thiophenecarboxylic acid		Xn; R22, R43

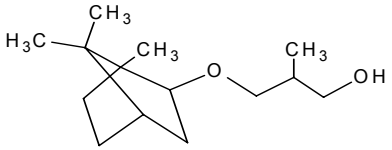
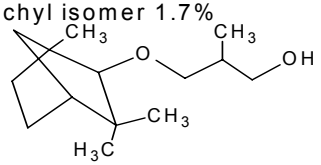
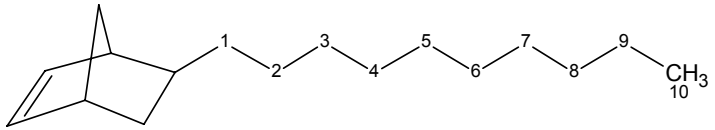
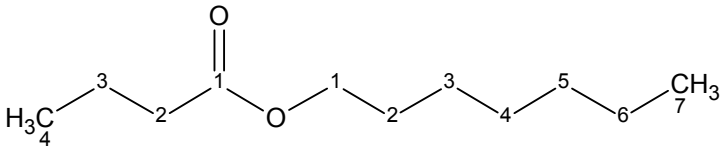
15 EU Non-Irritant GHS Non- Irritant	**EC#** **219-787-9** **CAS#** **2530-87-2** Liquid	**EINECS name** 3-chloropropyltrimethoxysilane **Synonym (SIVS)** silane A-1430		
16 EU Non-Irritant GHS Non- Irritant	**EC#** **203-953-2** **CAS#** **112-27-6** Liquid	**EINECS name** 2,2'-(ethylenedioxy)diethanol **Synonym (SIVS)** triethylene glycol		
17 EU Non-Irritant GHS Mild	**EC#** **416-350-6** **CAS#** **16947-63-0** 95-01-0346 Solid	**IUPAC name (NCD)** 2,6-dimethyl-4-nitrobenzeneamine		Xn; R22

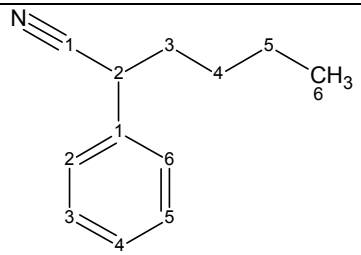
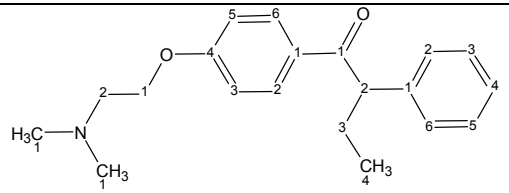
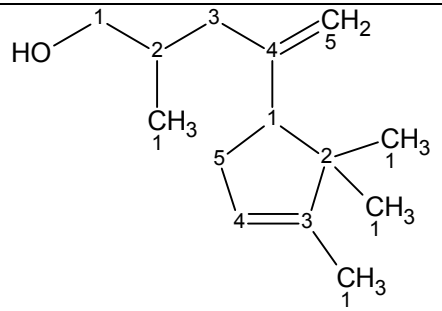
18 EU Non-Irritant GHS Non- Irritant	**EC#** **231-335-2** **CAS#** **7493-74-5** Liquid	**EINECS name** allyl phenoxyacetate		
19 EU Non-Irritant GHS Non- Irritant	**EC#** **200-661-7** **CAS#** **67-63-0** Liquid	**EINECS name** propan-2-ol **Synonym (SIVS)** isopropanol		F; R11 Xi; R36, R67 (Annex I)
20 EU Non-Irritant GHS Non- Irritant	**EC#** **420-170-3** **CAS#** **26218-04-2** 96-04-0805 Solid	**IUPAC name (Annex I)** 2-ethylhexyl 4-aminobenzoate		N; R50/53 (Annex I, Directive 67/548/EEC))

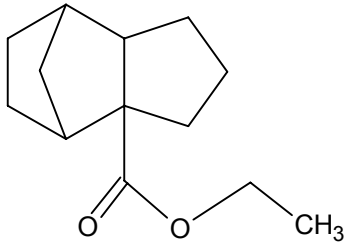
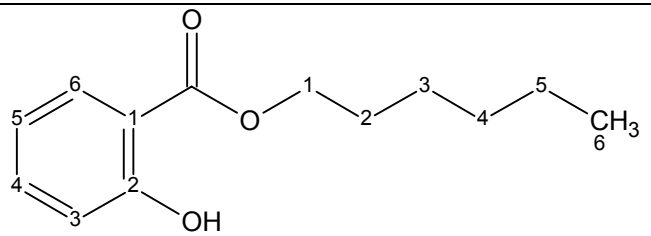
21 EU Non-Irritant GHS Non- Irritant	**EC#** **437-530-0** **CAS#** **0319002-92-1** 01-06-1517 Liquid	**IUPAC name (NCD)** propyl (2S)-2-(1,1-dimethylpropoxy)-propanoate		no symbol; R52/53
22 EU Non-Irritant GHS Non- Irritant	**EC#** **206-499-3** **CAS#** **350-30-1** Solid	**EINECS name** 2-chloro-1-fluoro-4-nitrobenzene **Synonym (SIVS)** 3-chloro-4-fluoronitrobenzene		
23 EU Non-Irritant GHS Non- Irritant	**EC#** **222-365-7** **CAS#** **3446-89-7** Liquid	**EINECS name** 4-(methylthio)-benzaldehyde		

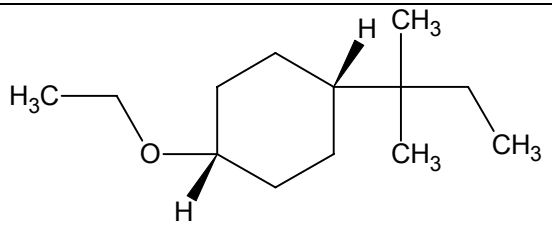
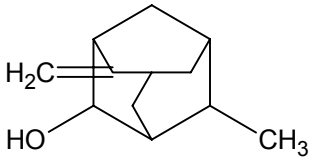
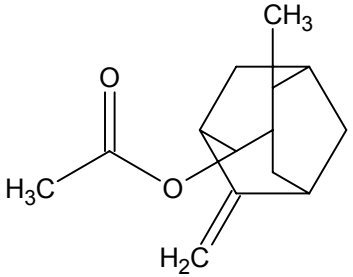
24 EU Non-Irritant GHS Non- Irritant	**EC#** **440-220-8** **CAS#** **209802-43-7** 02-01-0702 Liquid	**IUPAC name (NCD)** capryl-isostearate		no symbol; R53
25 EU Non-Irritant GHS Non- Irritant	**EC#** **203-990-4** **CAS#** **112-61-8** Solid	**EINECS name** methyl stearate		
26 EU Non-Irritant GHS Non- Irritant	**EC#** **200-456-2** **CAS#** **60-12-8** Liquid	**EINECS name** 2-phenylethanol **Synonym (SIVS)** phenylethylalcohol		

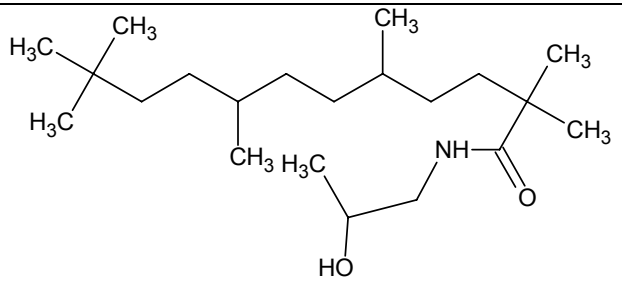
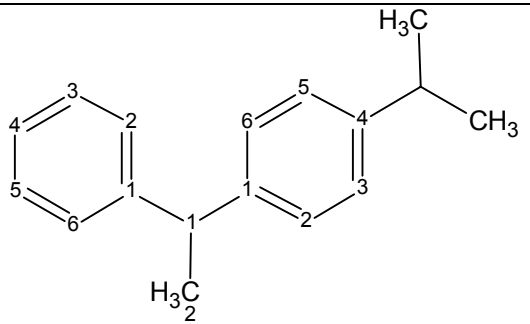
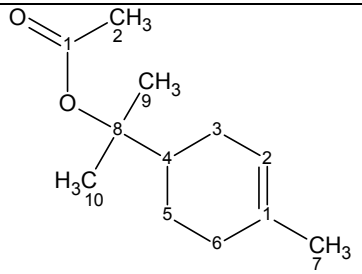
27 EU Non-Irritant GHS Non- Irritant	**EC#** **417-310-0** **CAS#** **0072903-27-6** 95-06-0733 Liquid	**IUPAC name (NCD)** Mixture of: diethyl cis-1,4-cyclohexanedicarboxylate; diethyl trans-1,4-cyclohexanedicarboxylate		N; R51/53
28 EU Non-Irritant GHS Non- Irritant	**EC#** **438-060-9** **CAS# (mix)** **224031-70-3** 02-06-1558 Liquid	**IUPAC name (NCD)** Mixture of isomers: 1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one (40-60%) 1-(spiro[4.5]dec-6-en-7-yl)pent-4-en-1-one (30-50%)		Xi; R43 N; R50/53
29 EU Non- Irritant GHS Mild	**EC#** **205-527-1** **CAS#** **142-19-8** Liquid	**EINECS name** allyl heptanoate		

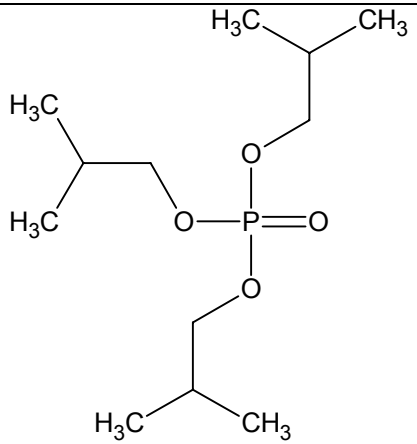
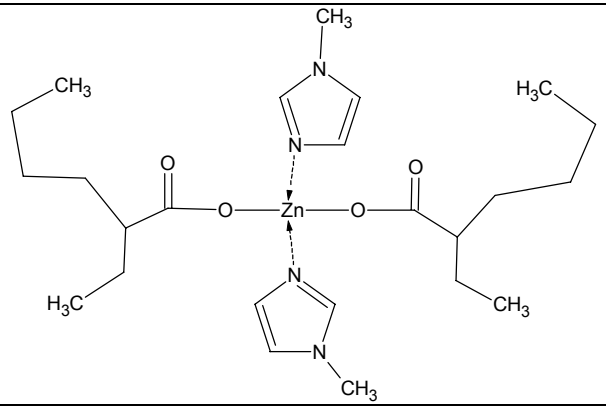
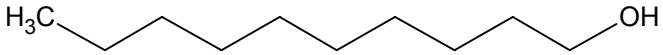
30 EU Non-Irritant GHS Mild	**EC#** **416-210-4** **CAS#** **128119-70-0** 95-07-0068 Liquid	**IUPAC name (NCD)** 2-methyl-3-[(1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, bornyl isomer **NB: Impurity (1.7%)** 2-methyl-3-[(1,3,3- trimethylbicyclo[2.2.1]hept-2-yl)oxy]-1-propanol, fenchyl isomer	Bornyl isomer 94.3%  Fenchyl isomer 1.7% 	Xi; R36 N; R51/53
31 EU Non-Irritant GHS Mild	**EC#** **435-040-1** **CAS#** **22094-85-5** 00-06-1442 Liquid	**IUPAC name (NCD)** Mixture of: 5-exo-decylbicyclo[2.2.1]hept-2-ene; 5-endo-decylbicyclo[2.2.1]hept-2-ene		Xn; R65 no symbol; R53
32 EU Non- Irritant GHS Mild	**EC#** **227-526-5** **CAS** **5870-93-9** Liquid	**EINECS name** heptyl butyrate		

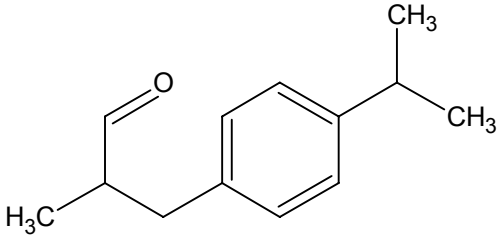
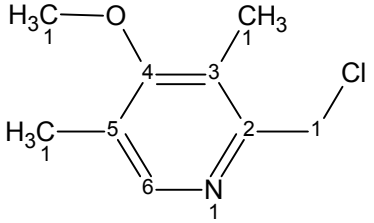
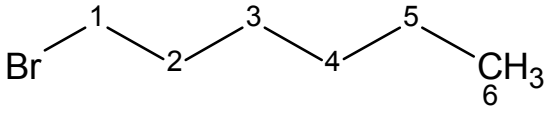
33 EU Non- Irritant GHS Mild	**EC#** **423-460-8** **CAS#** **3508-98-3** 97-11-0132 Liquid	**IUPAC name (Annex I)** 2-phenylhexanenitrile		Xn; R22 N; R50/53 (Annex I, Directive 67/548/EEC)
34 EU Irritant GHS Mild	**EC#** **426-600-6** **CAS#** **68047-07-07-4** 98-07-0153 Solid	**IUPAC name (ELINCS)** 1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenylbutan-1-one		Xi, Xn; R36/R38, R48/22, R62 N; R50/53
35 EU Irritant GHS Mild	**EC#** **435-750-1** **CAS#** **014864-90-6** 01-06-1480 Liquid	**IUPAC name (NCD)** Mixture of isomers: 2-methyl-4-(2',2',3'-trimethyl-3'-cyclopenten-1'-yl)-4-penten-1-ol 56% (1'R,2R) & 40%(1'R,2S) isomer		Xi; R38, R41 N; R50/53

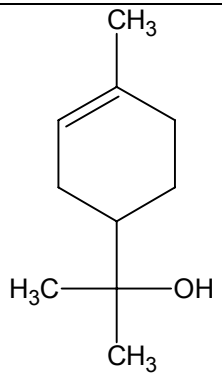
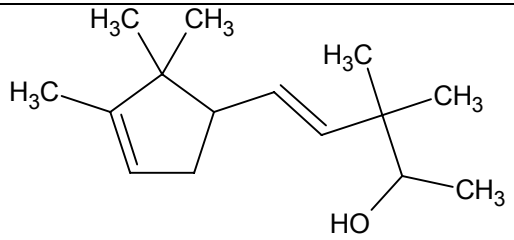
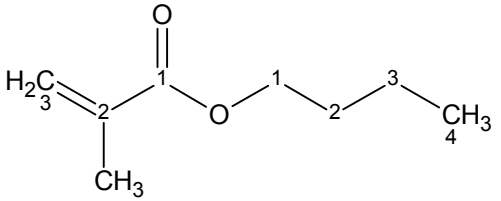
36 EU Irritant GHS Mild	**EC#** **407-520-0** **CAS#(mix)** **80657-64-3** 92-11-0044 Liquid	**IUPAC name (Annex I)** A mixture of: ethyl *exo*-tricyclo[5.2.1.$0^{2,6}$]decane-*endo*-2-carboxylate; ethyl *endo*-tricyclo[5.2.1.$0^{2,6}$]decane-*exo*-2-carboxylate Ratio of isomer 1: 35-60 Ratio of isomer 2: 40-65		Xi; R38 N; R51/53 (Annex I, Directive 67/548/EEC)
37 EU Irritant GHS Mild	**EC#** **228-408-6** **CAS#** **6259-76-3** Liquid	**EINECS name** hexyl salicylate		

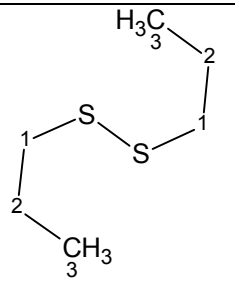
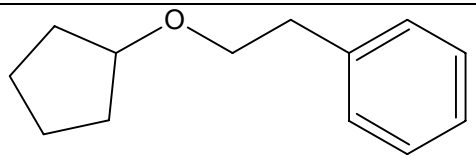
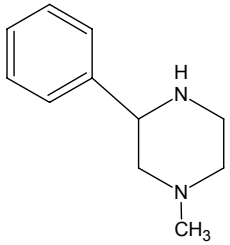
38 EU Irritant GHS Mild	**EC#** **426-530-6** **CAS#(cis)** **181258-87-7** **CAS#(trans)** **181258-89-9** 98-11-0150 Liquid	**IUPAC name (NCD)** Mixture of: 1-(1,1-dimethylpropyl)-4-ethoxy-cis-cyclohexane; 1-(1,1-dimethylpropyl)-4-ethoxy-trans-cyclohexane		Xi; R38 N; R50/53
39 EU Irritant GHS Mild	**EC#** **406-330-5** **CAS#** **122760-84-3** 90-03-0121 Solid	**IUPAC name (Annex I)** 4-methyl-8-methylenetricyclo[3.3.1.1^{3,7}]decan-2-ol		Xi; R38, R43 N; R51/53 (Annex I, Directive 67/548/EEC)
40 EU Irritant GHS Mild	**EC#** **406-560-6** **CAS#** **122760-85-4** 91-03-0162 Liquid	**IUPAC name (Annex I)** 4-methyl-8-methylenetricyclo[3.3.1.1^{3,7}]dec-2-yl acetate		Xi; R38, R43 N; R51/53 (Annex I, Directive 67/548/EEC)

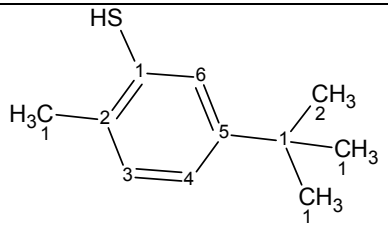
41 EU Irritant GHS Mild	**EC#** **431-540-9** **CAS#** **152848-22-1** 99-11-0170 Liquid	**IUPAC name (NCD)** isostearic acid monoisopropanolamide		Xi; R38 N; R51/53
42 EU Irritant GHS Mild	**EC#** **430-690-2** **CAS# (mix)** **52783-21-8** 99-06-1265 Liquid	**IUPAC name (NCD)** Mixture of isomers: 1-(2-isopropylphenyl)-1-phenylethane 1-(3-isopropylphenyl)-1-phenylethane 1-(4-isopropylphenyl)-1-phenylethane *(structure shown)*		Xi; R38 N; R50/53
43 EU Irritant GHS Mild	**EC#** **201-265-7** **CAS#** **80-26-2** Liquid	**EINECS name** p-menth-1-en-8-yl acetate **Synonym (SIVS)** terpinyl acetate		

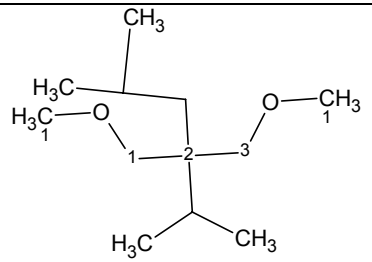
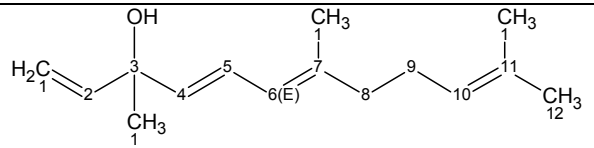
44 EU Irritant GHS Mild	**EC#** **204-798-3** **CAS#** **126-71-6** Liquid	**EINECS name** tri-isobutyl phosphate		
45 EU Irritant GHS Mild	**EC#** **405-635-0** **CAS#** **not allocated** 92-05-0165 Liquid	**IUPAC name (Annex I)** bis[(1-methylimidazol)-(2-ethyl-hexanoate)], zinc complex		Xi; R38, R41 N; R50/53 (Annex I, Directive 67/548/EEC)
46 EU/GHS Irritant	**EC#** **203-956-9** **CAS#** **112-30-1** Liquid	**EINECS name** decan-1-ol **Synonym (SIVS)** 1-decanol		

47 EU/GHS Irritant	**EC#** **203-161-7** **CAS#** **103-95-7** Liquid	**EINECS name** 3-p-cumenyl-2-methylpropionaldehyde **Synonym (SIVS)** cyclamen aldehyde		
48 EU/GHS Irritant	**EC#** **434-680-9** **CAS#** **86604-75-3** 00-11-0174 Solid	**IUPAC name (NCD)** 2-chloromethyl-3,5-dimethyl-4-methoxypyridine hydrochloride	HCl 	Xn; R22, R38, R41 N; R50/53
49 EU/GHS Irritant	**EC#** **203-850-2** **CAS#** **111-25-1** Liquid	**EINECS name** 1-bromohexane		

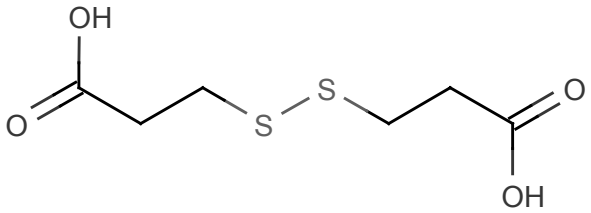
50 EU/GHS Irritant	**EC#** **202-680-6** **CAS#** **98-55-5** Liquid	**EINECS name** p-menth-1-en-8-ol **Synonym (SIVS)** α-terpineol		
51 EU/GHS Irritant	**EC#** **411-580-3** **CAS#** **107898-54-4** 92-06-0400 Liquid	**IUPAC name (Annex I)** (+/-) *trans*-3,3-dimethyl-5-(2,2,3-trimethyl-cyclopent-3-en-1-yl)-pent-4-en-2-ol		Xi; R38 N; R50/53 (Annex I, Directive 67/548/EEC)
52 EU/GHS Irritant	**EC#** **202-615-1** **CAS#** **97-88-1** Liquid	**IUPAC name (Annex I)** n-butyl methacrylate **Synonym (SIVS)** butyl methacrylate		Xi; R36/37/38, R43 no symbol; R10 (Annex I, Directive 67/548/EEC)

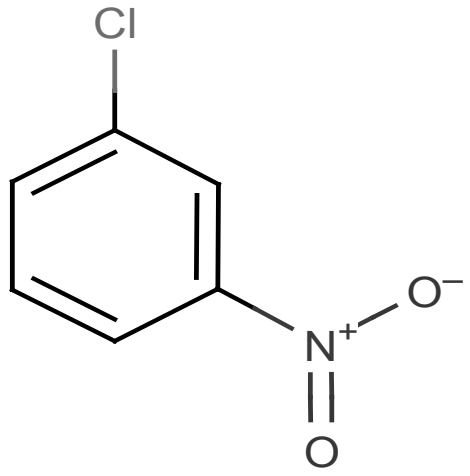
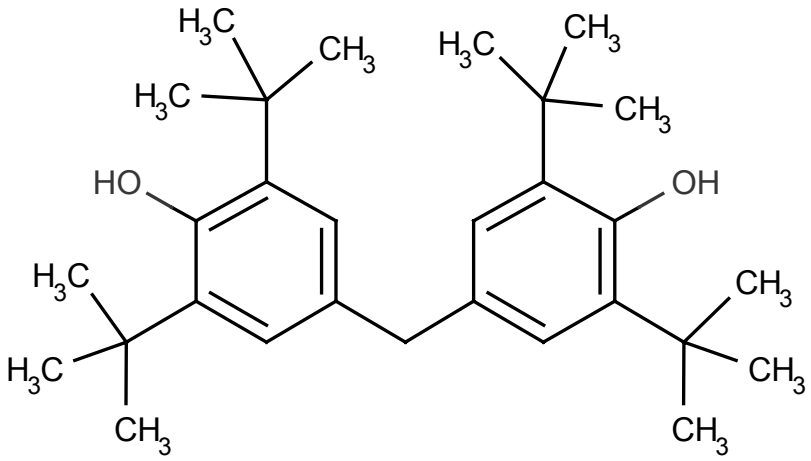
53 EU/GHS Irritant	**EC#** **211-079-8** **CAS#** **629-19-6** Liquid	**EINECS name** dipropyl disulphide **Synonym (SIVS)** di-n-propyl disulphide		
54 EU/GHS Irritant	**EC#** **428-340-9** **CAS#** **not allocated** 98-03-0429 Liquid	**IUPAC name (NCD)** [2-(cyclopentyloxy)ethyl]benzene (cyclopentyl 2-phenylethyl ether)		Xi; R38 N; R50/53
55 EU/GHS Irritant	**EC#** **431-180-2** **CAS#** **5271-27-2** 99-06-1280 Solid	**IUPAC name (NCD)** 1-methyl-3-phenyl-1-piperazine		Xn; R21/22, R38, R41 no symbol; R52/53

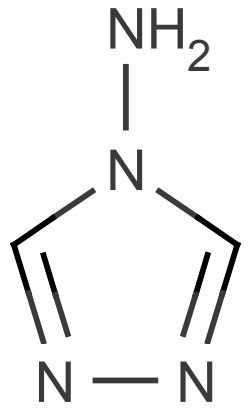
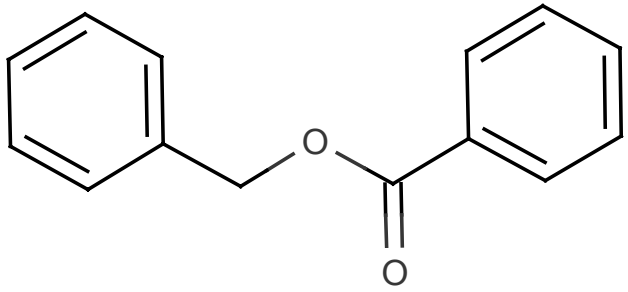
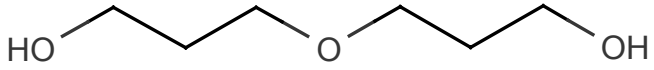
56 EU/GHS Irritant	**EC#** **438-520-9** **CAS#** **7340-90-1** 99-01-0585 Liquid	**CAS name (SIVS)** benzenethiol, 5-(1,1-dimethylethyl)-2-methyl		Xn; R36, R38, R43, R48/22 N; R50/53

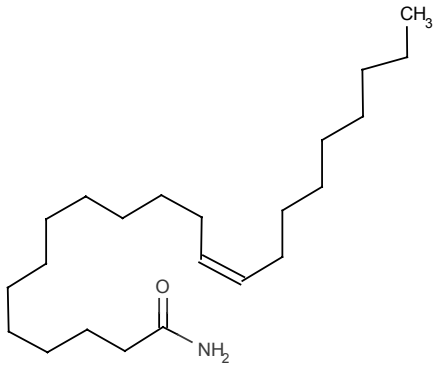
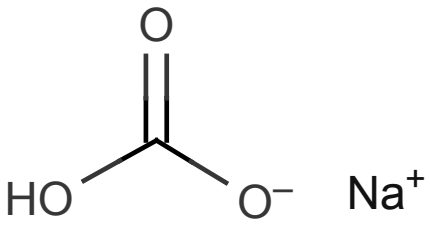
57 EU/GHS Irritant	**EC#** **430-800-9** **CAS#** **129228-21-3** 99-05-0348 Liquid	**IUPAC name (NCD)** 2-isopropyl-2-isobutyl-1,3-dimethoxypropane		Xi; R38 N; R51/53
EU/GHS Irritant	**EC#** **423-240-1** **CAS#** **125474-34-2** 96-01-0420 Liquid	**IUPAC name (NCD)** (E,E)-3,7,11-trimethyldodeca-1,4,6,10-tetraen-3-ol		Xi; R38, R41, R43 N; R50/53

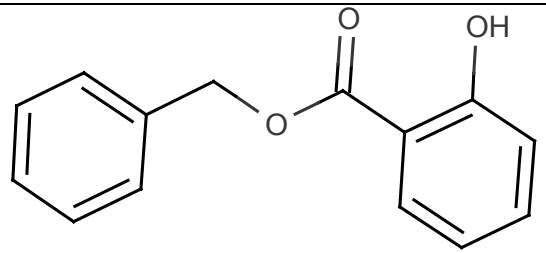
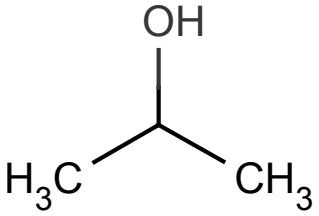
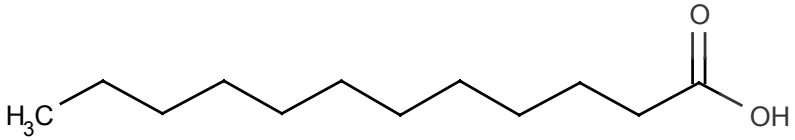
ANNEXE 9 – CHEMICAL STRUCTURES OF THE TEST SUBSTANCES USED DURING THE OPTIMISATION PHASES

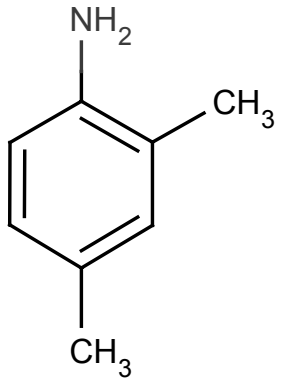
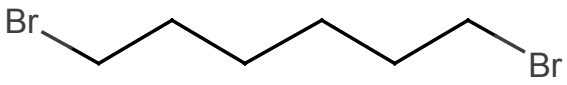
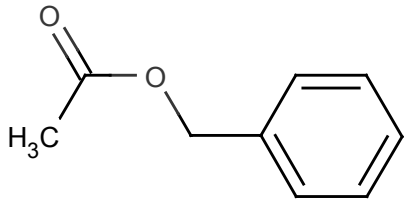
1 **NAME: Propanoic acid, 3,3'-dithiobis-** **RN: 1119-62-6** **Systematic Name** 3,3'-Dithiobispropionic acid Propanoic acid, 3,3'-dithiobis- Propionic acid, 3,3'-dithiodi- (8CI)	

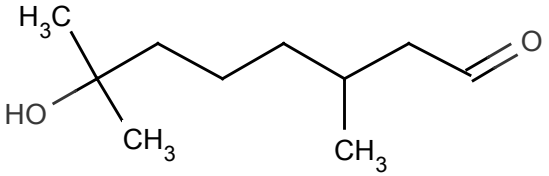
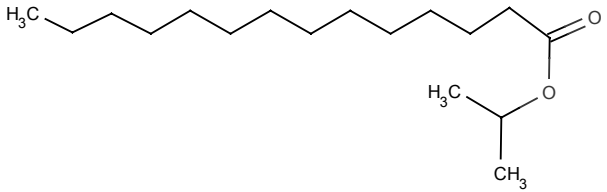
2 **NAME: 1-Chloro-3-nitrobenzene** **RN: 121-73-3** **Systematic Name** 1-Chloro-3-nitrobenzene Benzene, 1-chloro-3-nitro- m-Chloronitrobenzene	
3 **NAME: 4,4'-Methylenebis(2,6-di-tert-butylphenol)** **RN: 118-82-1** **Systematic Name** 2,2',6,6'-Tetra-tert-butyl-4,4'-methylenediphenol 4,4'-Methylenebis(2,6-di-tert-butylphenol) Phenol, 4,4'-methylenebis(2,6-bis(1,1-dimethylethyl)- Phenol, 4,4'-methylenebis(2,6-di-tert-butyl- (8CI)	

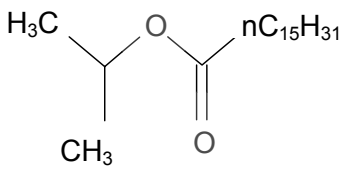
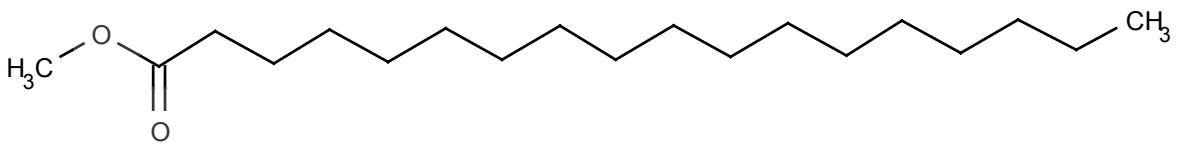
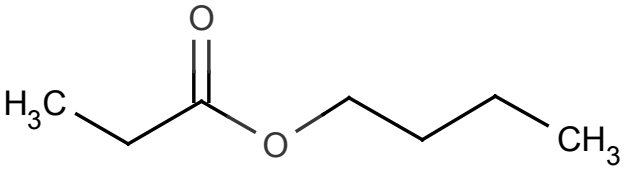
4 **NAME: 4H-1,2,4-Triazole, 4-amino-** **RN: 584-13-4** **Systematic Name** 4H-1,2,4-Triazol-4-amine 4H-1,2,4-Triazol-4-ylamine 4H-1,2,4-Triazole, 4-amino-	
5 **NAME: Benzyl benzoate [USAN:JAN]** **RN: 120-51-4** **Name of Substance** **Systematic Name** Benzoic acid, benzyl ester Benzoic acid, phenylmethyl ester Benzyl benzoate Benzylbenzenecarboxylate	
6 **NAME: Dipropylene glycol** **RN: 25265-71-8** **Systematic Name**	

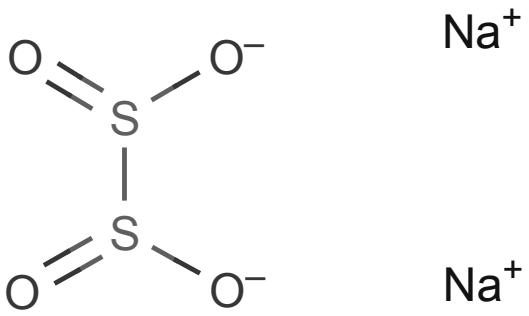
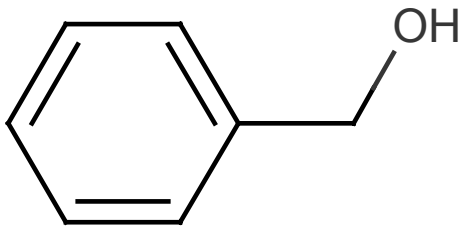
Dipropylene glycol Oxydipropanol Propanol, oxybis-	
7 **NAME: Erucamide** **RN: 112-84-5** **Systematic Name** (Z)-Docos-13-enamide 13-Docosenamide, (13Z)- 13-Docosenamide, (Z)-	
8 **NAME: Sodium bicarbonate [USAN:JAN]** **RN: 144-55-8** **Systematic Name** Carbonic acid monosodium salt Carbonic acid sodium salt (1:1) Sodium bicarbonate Sodium bicarbonate (1:1) Sodium hydrogencarbonate	

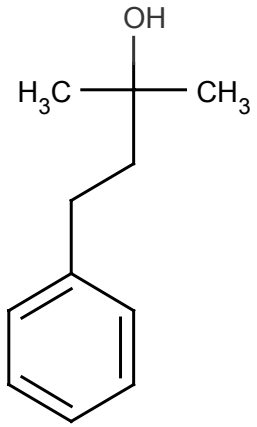
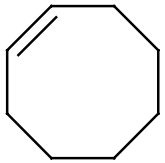
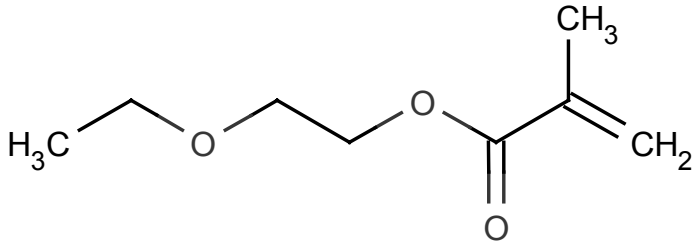
9 **NAME: Benzoic acid, 2-hydroxy-, phenylmethyl ester** **RN: 118-58-1** **Systematic Name** Benzoic acid, 2-hydroxy-, phenylmethyl ester Benzyl salicylate Salicylic acid, benzyl ester	
10 **NAME: Isopropyl alcohol [USAN]** **RN: 67-63-0** **Systematic Name** 2-Propanol Isopropyl alcohol	
11 **NAME: Dodecanoic acid** **RN: 143-07-7** **Systematic Name** Dodecanoic acid Lauric acid Lauric acid, pure	

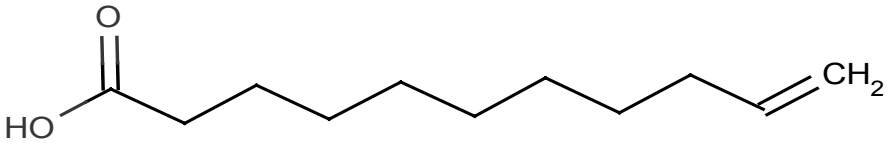
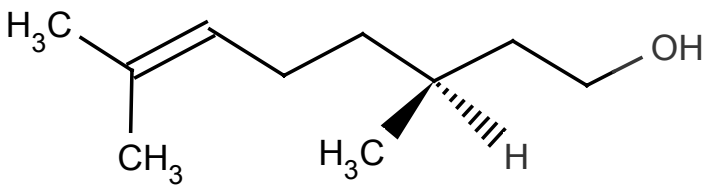
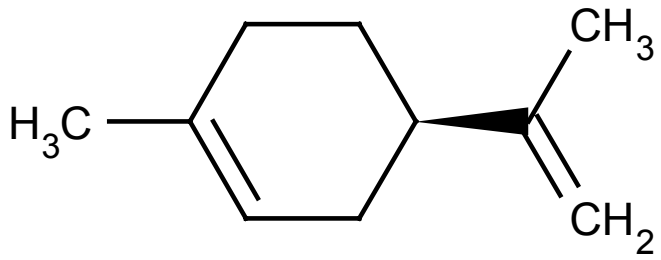
12 **NAME: 2,4-Xylidine** **RN: 95-68-1** **Systematic Name** 2,4-Dimethylaniline 2,4-Xylidine Benzenamine, 2,4-dimethyl-	
13 **Soap from 20/80 coconut oil/tallow**	
14 **NAME: Hexane, 1,6-dibromo-** **RN: 629-03-8** **Systematic Name** 1,6-Dibromohexane Hexane, 1,6-dibromo-	
15 **NAME: Benzyl acetate** **RN: 140-11-4** **Systematic Name** Acetic acid, benzyl ester Acetic acid, phenylmethyl ester	

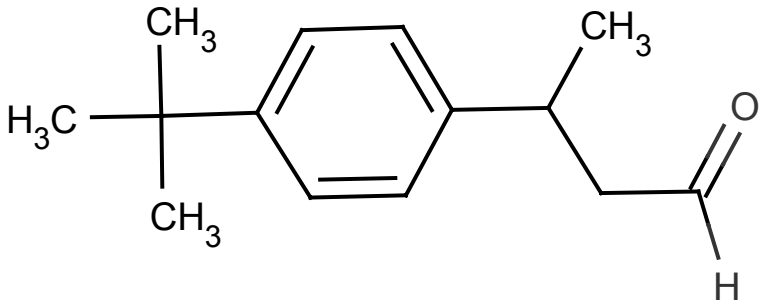
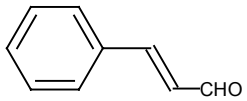
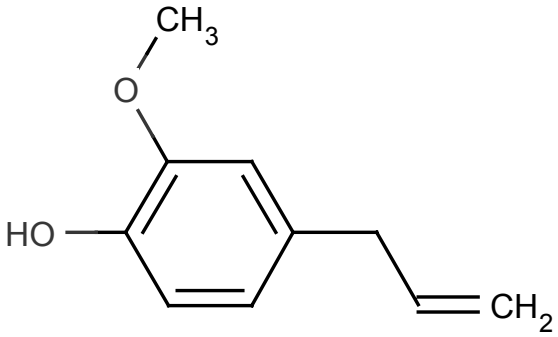
Benzyl acetate	
16 **NAME: Hydroxycitronellal** **RN: 107-75-5** **Systematic Name** 1-Octanal, 3,7-dimethyl-7-hydroxy- 7-Hydroxy-3,7-dimethyloctanal 7-Hydroxycitronellal Octanal, 7-hydroxy-3,7-dimethyl-	
17 **NAME: Isopropyl myristate [USAN]** **RN: 110-27-0** **Systematic Name** Isopropyl myristate Myristic acid, isopropyl ester (8CI) Tetradecanoic acid, 1-methylethyl ester Tetradecanoic acid, isopropyl ester	

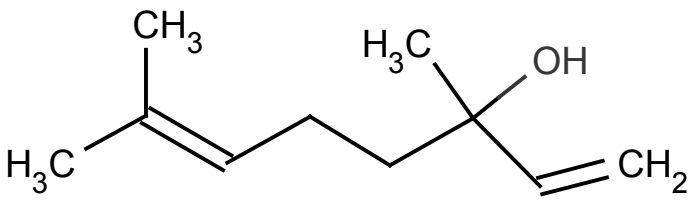
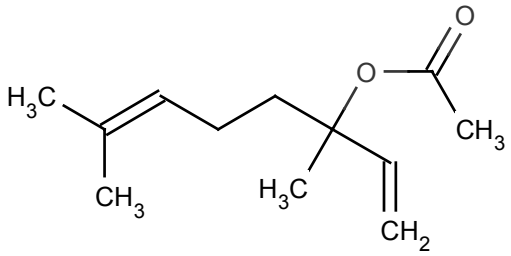
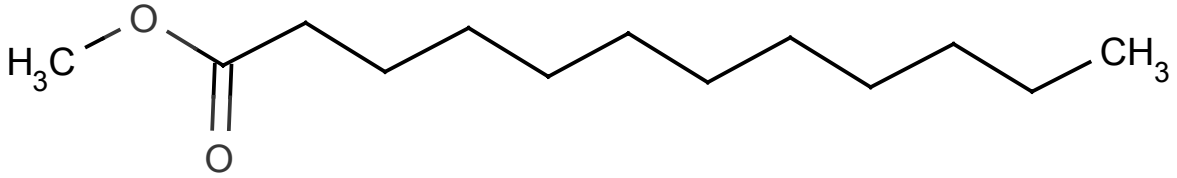
18 **NAME: Isopropyl palmitate** **RN: 142-91-6** **Systematic Name** ℹHexadecanoic acid, 1-methylethyl ester ℹIsopropyl palmitate ℹPalmitic acid, isopropyl ester	
19 **NAME: Methyl stearate** **RN: 112-61-8** **Systematic Name** ℹMethyl stearate ℹOctadecanoic acid, methyl ester ℹStearic acid, methyl ester	
20 **NAME: n-Butyl propionate** **RN: 590-01-2** **Systematic Name** ℹButyl propionate ℹPropanoic acid, butyl ester ℹPropionic acid, butyl ester	

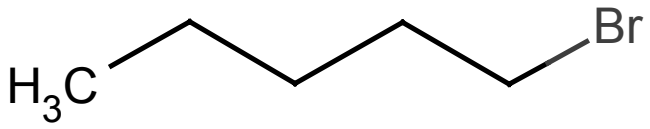
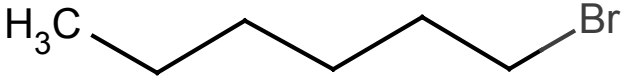
21 **NAME: Sodium hydrosulfite** **RN: 7775-14-6** **Systematic Name** Dithionous acid, disodium salt Dithionous acid, sodium salt (1:2) Sodium dithionite Sodium hydrosulfite	
22 **NAME: Benzyl alcohol [USAN:INN:JAN]** **RN: 100-51-6** **Systematic Name** Benzenemethanol Benzyl alcohol	

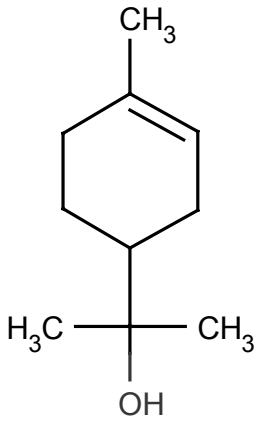
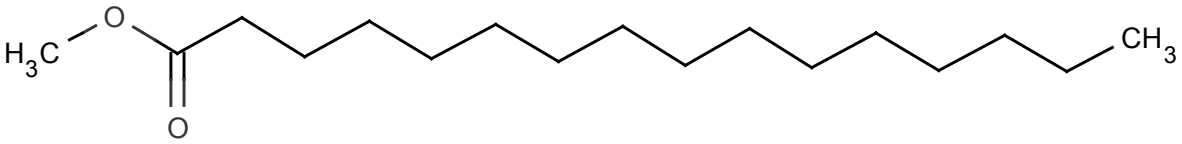
23 **NAME: Benzenepropanol, alpha,alpha-dimethyl-** **RN: 103-05-9** **Systematic Name** 1-Propanol, 1,1-dimethyl-3-phenyl- (8CI) 2-Butanol, 2-methyl-4-phenyl- 2-Methyl-4-phenylbutan-2-ol Benzenepropanol, alpha,alpha-dimethyl-	
24 **NAME: Cyclooctene, (1Z)-** **RN: 931-87-3** **Systematic Name** (Z)-Cyclooctene Cyclooctene, (1Z)-	
25 **NAME: 2-Ethoxy ethyl methacrylate** **RN: 2370-63-0** **Systematic Name** 2-Ethoxyethyl methacrylate 2-Propenoic acid, 2-methyl-, 2-ethoxyethyl ester	

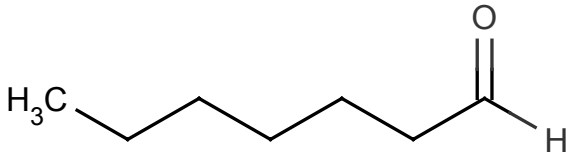
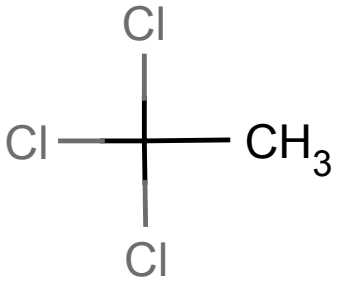
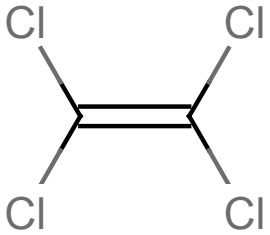
26 **NAME: Amines, N-tallow alkyltripropylenetetra-** **RN: 68911-79-5** **Systematic Name** Amines, N-tallow alkyltripropylenetetra-	No structure
27 **NAME: Undecylenic acid [JAN]** **RN: 112-38-9** **Systematic Name** 10-Undecenoic acid	
28 **NAME: Citronellol** **RN: 106-22-9** **Systematic Name** 3,7-Dimethyl-6-octen-1-ol 6-Octen-1-ol, 3,7-dimethyl- Citronellol	
29 **NAME: (d)-Limonene** **RN: 5989-27-5** **Systematic Name** (R)-p-Mentha-1,8-diene Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (4R)- Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (R)- Cyclohexene, 1-methyl-4-(1-	

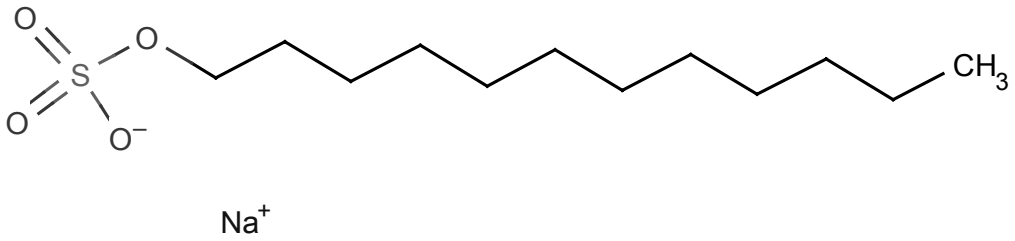
methylethenyl)-, (theta)- ℹD-Limonene	
30 **NAME: Lilial** **RN: 80-54-6** **Systematic Name** ℹ2-(4-tert-Butylbenzyl)propionaldehyde ℹBenzenepropanal, 4-(1,1-dimethylethyl)-alpha-methyl- ℹHydrocinnamaldehyde, p-tert-butyl-alpha-methyl-	
31 **NAME: Cinnamaldehyde** **RN: 104-55-2** **Systematic Name** ℹ2-Propenal, 3-phenyl- ℹCinnamaldehyde	
32 **NAME: Eugenol [USAN]** **RN: 97-53-0** **Systematic Name** ℹEugenol ℹPhenol, 2-methoxy-4-(2-propen-1-yl)- ℹPhenol, 2-methoxy-4-(2-propenyl)- ℹPhenol, 4-allyl-2-methoxy-	

33 **NAME: Linalyl alcohol** **RN: 78-70-6** **Systematic Name** (1)-3,7-Dimethyl-1,6-octadien-3-ol 1,6-Octadien-3-ol, 3,7-dimethyl- 3,7-Dimethyl-1,6-octadien-3-ol Linalool	
34 **NAME: Linalyl acetate** **RN: 115-95-7** **Systematic Name** (1)-1,5-Dimethyl-1-vinylhex-4-enyl acetate 1,6-Octadien-3-ol, 3,7-dimethyl-, 3-acetate 1,6-Octadien-3-ol, 3,7-dimethyl-, acetate Linalyl acetate	
35 **NAME: Methyl dodecanoate** **RN: 111-82-0** **Systematic Name** Dodecanoic acid, methyl ester	

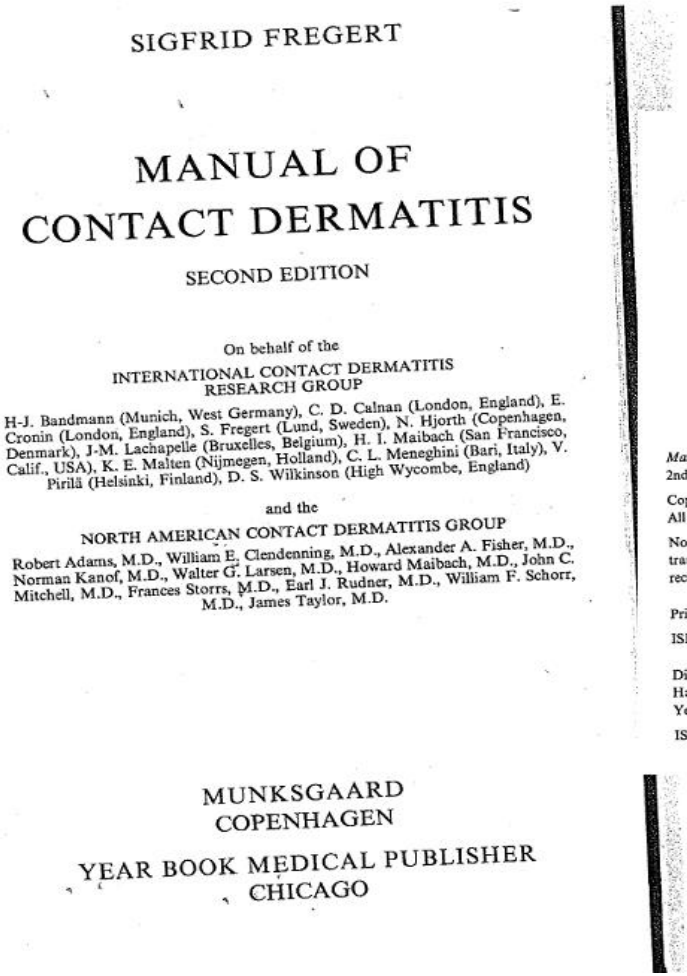
Lauric acid, methyl ester Methyl laurate	
36 **NAME: n-Amyl bromide** **RN: 110-53-2** **Systematic Name** 1-Bromopentane Pentane, 1-bromo-	
37 **NAME: Hexane, 1-bromo-** **RN: 111-25-1** **Systematic Name** 1-Bromohexane Hexane, 1-bromo-	

38 **NAME: alpha-Terpineol** **RN: 98-55-5** **Systematic Name** (1)-alpha,alpha,4-Trimethylcyclohex-3-ene-1-methanol 3-Cyclohexene-1-methanol, alpha,alpha,4-trimethyl- alpha,alpha,4-Trimethyl-3-cyclohexene-1-methanol alpha-Terpineol p-Menth-1-en-8-ol	
39 **NAME: Methyl palmitate** **RN: 112-39-0** **Systematic Name** Hexadecanoic acid, methyl ester Methyl palmitate Palmitic acid, methyl ester (8CI)	
40 **NAME: Potassium hydroxide [JAN]** **RN: 1310-58-3** **Systematic Name**	

Potassium hydroxide Potassium hydroxide (K(OH))	
41 **NAME: N-Heptanal** **RN: 111-71-7** **Systematic Name** Heptanal N-Heptanal	
42 **NAME: Methylchloroform** **RN: 71-55-6** **Systematic Name** 1,1,1-Trichloroethane Ethane, 1,1,1-trichloro-	
43 **NAME: Tetrachloroethylene** **RN: 127-18-4** **Systematic Name** 1,1,2,2-Tetrachloroethylene Ethene, 1,1,2,2-tetrachloro- Ethene, tetrachloro- Ethylene, tetrachloro- Tetrachloroethylene	

44 **NAME: Sodium Lauryl Sulfate [USAN:JAN]** **RN: 151-21-3** **Systematic Name** Sodium dodecyl sulfate Sodium dodecyl sulphate Sulfuric acid monododecyl ester sodium salt Sulfuric acid monododecyl ester sodium salt (1:1)	

ANNEXE 10 – COVER INFORMATION AND PAGES 63 – 67 ON "COMMON IRRITANTS" FROM THE PUBLICATION BY FREGERT (1981)



SIGFRID FREGERT

MANUAL OF CONTACT DERMATITIS

SECOND EDITION

On behalf of the

INTERNATIONAL CONTACT DERMATITIS RESEARCH GROUP

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8. Common irritants

8.1 Water

If the surface film and horny layer have been damaged by solvents, detergents, etc., water can dissolve the water-binding substances in the horny layer and cause dryness. Water is hypotonic and can be toxic to the living epidermal cells. Calcium, magnesium and iron in hard water can be deposited in the skin cracks and cause mechanical and chemical irritation. High concentrations of chlorine in swimming pools can irritate. If the water is permitted to act for a long time a maceration takes place and the possibilities for harmful substances to penetrate are increased.

8.2 Skin cleansers

Soap does not seem harmful to normal skin, but if the skin has previously been damaged, the soap can have an irritant effect. Abrasives in soaps often have more damaging effect than the soap itself. Certain so-called water-free cleansers containing solvents have a more powerful effect on the skin. In many occupations where the skin gets dirty, the cleansers – especially in the form of organic solvents or alkalis – cause considerably greater damage to the skin than the work itself.

8.3 Cleansers

Detergents and dish – washing liquids in concentrations prescribed by the manufacturer have a fairly mild effect on normal skin. Most often such substances are used in too high a concentration in the

suds. When detergents as powder are added to washing machines and dishwashers, the hands are easily contaminated. They work first by dissolving fat and water-binding substances. When they penetrate deeper, they denature the protein and damage cell membranes. Proteolytic enzymes in detergents can irritate. Certain perfumes in detergents may act as irritants when dissolved in hot water.

8.4 Alkalis

The most common are soap, soda, ammonium, potassium and sodium hydroxide, cement, lime, sodium silicate, trisodium phosphate and different types of amine (e.g. epoxy resin hardeners), stearylamine (emulsifier), monoethanol amine in antirust agents. They dissolve the skin's fat and water binding substances and break the chemical chains in the keratin. Alkalis are used in many industries, such as dyeing, tanning, and manufacturing of plastics and glass. Certain copying processes use ammonium, which is vaporized and can cause irritation on the face. Soda ash (anhydrous sodium carbonate) is three times stronger than common washing soda.

8.5 Acids

Diluted acids generally damage the skin less than the corresponding concentrations of alkalis. Strong concentrations of, e.g. hydrochloric acid, nitric acid, chromic acid, hydrofluoric acid, are used in many industries and can cause corrosion of the skin. Organic acids (e.g. oxalic acid) are present in some plants and bulbs. Among saturated free fatty acids, particularly C_8 through C_{12} are irritating.

8.6 Oils

Soluble cutting oils and coolants contain oil, water and emulsifiers and also antioxidants, anticorrosion agents, preservatives and sometimes perfume. Through their emulsifying effect they dry out the skin. Sulfated and chlorinated oils are more irritating.

Lubricating oils and hydraulic oils are generally difficult to remove. The consequence is that one is often forced to use organic solvents to get the hands clean, which can further worsen the injury.

8.7 Organic solvents

These are extensively used in many industries. The most common are white spirit, benzene, toluene, aromatic petroleum solvents, trichloroethylene (TRI), perchlorethylene (PER), methylene chloride, trichlorethane (methylchloroform) chlorobenzene, methanol, ethanol, isopropanol, propylene glycol, ethylacetate, acetone, methylethylketone, nitroethane, and carbon disulfide.

Thinner often consists of a mixture of alcohols, ketones and sometimes toluene and dipentene (sensitizer). The aromatic solvents especially irritate the skin. White spirit (kerosene) contains 18% aromatics and primegrade kerosene 2%, sometimes down to 0.1%.

In cleansing from oil, a mixture of solvents and detergents is used. Solvents which are not completely removed from for example working clothing after waterless cleansing can irritate the skin on legs, wrists and neck.

8.8 Oxidants

Hydrogen peroxide and especially organic peroxides, e.g. benzoyl peroxide and cyclohexanon peroxide, are used in several industries including the production of polyester plastic products. Organic peroxides may occur in flour and perborate in washing powders.

8.9 Reducing agents

Thioglycolates are used in permanent waving of hair. They break the chemical links between the keratin molecules, allowing the keratin to swell and thereby increase the penetration possibilities.

8.10 Plants

Orange peel and hyacinth and tulip bulbs contain irritants, as do

pineapple juice, cucumbers, asparagus, mustard, barley and corn, spurge, pasque flower, wind flower, rice, bamboo.

8.11 Animal substances

Enzymes which come onto the skin when the pancreas is being removed from the intestines in slaughterhouses can damage the skin and cause onycholysis. Feces, urine and the intestinal contents from colostomies can have an irritant effect. Fish and shrimp are irritating. Butchers develop irritation on the forearms due to friction of the pigs' skin and possibly fatty acids.

8.12 Other organic substances

Formaldehyde, allyl alcohol, cyanoacrylate in glue, cresol, chlorocresols and -phenols, occur in pesticides, alkyl bromides in pain killers, styrene in plastic monomers and halogenated acetophenones in tear gas (Mace). Alkyl-tin compounds (TBTO) are used as preservatives for wood and textiles.

Compounds with unsaturated chains are irritants, e.g. allyl alcohol and -aldehyde, diallyl-phthalate and -glycol and crotonaldehyde.

8.13 Medicaments for local treatment

Tar, potassium permanganate, gentian violet, hexachlorophene, quaternary ammonium compounds, mercury preparations, anthralin can – especially if they are used in high concentrations – cause "overtreatment dermatitis". Occlusive treatment magnifies this potential.

8.14 Other inorganic substances

Bromine, chlorine, mercury salts and zinc chlorides or phosphoric acid in solders, sodium bifluoride in flux and wood preservatives and antimony trioxide are irritants.

8.15 Physical and mechanical factors

Heat, steam, cold, sunshine, ultraviolet light and other radiations are irritating to the skin. Friction, pressure and scratching of the skin can cause increased permeability. Metal particles, adhesive plaster, insulating tape, glass wool, particles of textiles, sawdust, sand, asbestos, cement and plaster can cause an increase in permeability mechanically.

8.16 Cosmetics

Mascara preparations, face creams, antiperspirants (aluminum chlorhydroxide is less irritant than aluminum chloride), deodorants with quaternary ammonium compounds, propellants in hygiene sprays, cleaning solutions, may be irritants. Propylene glycol is an irritant.

8.17 Carbonless paper (NCR paper)

NCR (No Carbon Required, National Cash Register) can cause irritation on the face and the hands/arms probably owing to solvents released when the small capsules containing dye are broken at writing.

8.18 Plastic low molecular material

Several monomers and cross-linking substances are sensitizers and irritants. Particularly irritating are acrylates, phenol-formaldehyde resins, isocyanates, diallylphthalate, diallyl glycol carbonate, styrene. The epoxy oligomers of bisphenol A type are weak or non irritants.

ANNEXE 11 – THE DERMAL TOXICITY TESTING STRATEGY OF TG 404

Annex 11 - Predicting dermal toxicity using the TG 404 integrated testing strategy – a way of excluding false negative predictions

Contributed by Helena Kandárová, MatTek Corporation, Ashland MA, USA and Manfred Liebsch, ZEBET at the BfR Berlin, Germany; edited by Claudius Griesinger and Joao Barroso, EC-JRC, Ispra, Italy.

February 2010

1. Summary

The TG 404 test strategy is presented in this Annex to address the input of one member country made during one of the OECD commenting rounds pertaining to the draft OECD TG on *in vitro* skin irritation testing (December 2009).

The question put forward concerned corrosive substances that were falsely predicted as negatives ("false negative corrosives") by the validated **skin corrosion** test methods. These false negatives had been compiled in a list by ICCVAM/NICEATM. In particular the concern was whether the present validated skin irritation test methods would pick up these false negatives as, at least, skin irritants.

Despite the fact that this issue is beyond the scope of the irritation test methods, which were designed and validated to distinguish between chemicals considered non-irritant and irritant, the present paper demonstrates that all false negatives that may possibly exit from the *in vitro* skin corrosion tests alone are further excluded (i.e. detected as positives / corrosives) if the entirety of the TG 404 testing strategy is properly applied, i.e. using – inter alia - QSAR analysis, pH measurement, measurement of acid-alkaline reserve, evaluation of data from acute toxicity via the dermal route *in vivo*, analysis of available human data. Furthermore, it should be noted that all the commercially available substances from the NICEATM/ICCVAM list are predicted as irritants by the 60min/42h EpiDerm^{TM} *in vitro* skin irritation test.

This document demonstrates that, on the basis of the empirical data available at this point, the testing strategy outlined in the appendix of TG 404 (i.e. the combined sequential use of various information sources including *in vitro* skin corrosion and skin irritation tests) provides adequate information for decision-making on potential hazards of chemicals to the skin.

2. Introduction

An effective way of predicting chemical toxicity while reducing animal testing is to make use of testing strategies which incorporate a range of alternative methods, and which resort to animal tests only when necessary ***(1)***. A stepwise (hierarchical) testing strategy for the prediction of skin irritation and corrosion was developed (**Figure A11.1**) and unanimously recommended by the participants of an OECD workshop on *Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods,* held in Solna, Sweden, in January 1996 ***(2)*** and has been adopted as the recommended testing strategy in the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) ***(3, 4)***. The strategy is also annexed to the OECD TG 404 ***(5)*** which states:

„In the interest of both sound science and animal welfare, in vivo testing should not be undertaken until all available data relevant to the potential dermal corrosivity/irritation of the substance have been evaluated in a weight-of-the-evidence analysis. Such data will include evidence from existing studies in humans and/or laboratory animals, evidence of corrosivity/irritation of one or more structurally related substances or mixtures of such substances, data demonstrating strong acidity or alkalinity of the substance, and results from validated and accepted in vitro or ex vivo tests. This analysis should decrease the need for in vivo testing for dermal corrosivity/irritation of substances for which sufficient evidence already exists from other studies as to those two endpoints".

Although this sequential testing strategy is not an integral part of Test Guideline 404, it expresses the recommended approach for the determination of skin irritation/corrosion characteristics. This approach represents best practice, as accepted by international consensus at present, and provides a rational framework for the assessment on whether or not *in vivo* testing is required to fulfill data requirements for skin irritation/corrosion ***(5)***. Moreover, the strategy also served as basis for the Guidance Document for Industry on Information Requirements under REACH (for the human health effect skin irritation/corrosion) in Europe (available on ECHA's website: http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm?time=1262869754).

3. Results

When applying the above mentioned testing strategy (described below) on the substances identified by NICEATM/ICCVAM as potentially false negative corrosives (**Table A11.1**) ***(6)***, all these substances are correctly identified as corrosive, and the number of false negative predictions decreases to zero (**Table A11.2**).

Dermal toxicity LD50 data are also available (**Table A11.3**) for most of the substances listed in Table A11.1, which could be used in Step 4 of the testing strategy described in OECD TG 404 (please see below). These data would further contribute to correctly classify and label all substances listed in Table A11.1.

Moreover, it should be noted that the validated RhE-based *in vitro* corrosion test methods will properly identify most of the skin corrosives listed in Table A11.1 ***(6)*** on their own, if applying proper controls for direct MTT reduction (**Table A11.2**).

Finally, the validated 60min/42h EpiDerm^{TM} skin irritation test predicts all the commercially available corrosive substances from the NICEATM/ICCVAM list as skin irritants ***(7)***, and thus, it is expected that other RhE-based skin irritation tests would also pick up the false negatives identified by NICEATM/ICCVAM as, at least, skin irritants.

4. Description of the Test Strategy

See Figure A11.1

Step 1 - Evaluation of existing human and animal data. Existing human data, e.g. clinical or occupational studies and case reports, and/or animal test data, e.g. from single or repeated dermal exposure toxicity studies, should be considered first, because they provide information directly related to effects on the skin. Substances with known irritancy or corrosivity, and those with clear evidence of non-corrosivity or non-irritancy, need not be tested in *in vivo* studies.

Step 2 - Analysis of structure activity relationships. The results of testing of structurally related substances should be considered, if available. When sufficient human and/or animal data are available on structurally related substances or mixtures of such substances to indicate their skin corrosion/irritancy potential, it can be presumed that the test substance being evaluated will produce the same responses. In those cases, the test substance may not need to be tested. Negative data from studies of structurally related substances or mixtures of such substances do not constitute sufficient evidence of noncorrosivity/ non-irritancy of a substance under the sequential testing strategy. Validated and accepted SAR approaches should be used to identify both dermal corrosion and irritation potential.

Step 3 - Physicochemical properties and chemical reactivity. Substances exhibiting pH extremes such as ≤2.0 and ≥11.5 may have strong local effects. If extreme pH is the basis for identifying a substance as corrosive to skin, then its acid/alkali reserve (or buffering capacity) may also be taken into consideration. If the buffering capacity suggests that a substance may not be corrosive to the skin, then further testing should be undertaken to confirm this, preferably by the use of a validated and accepted *in vitro* or *ex vivo* test.

Step 4 - Dermal toxicity. If a chemical has proven to be highly toxic by the dermal route, an *in vivo* dermal irritation/corrosion study may not be practicable because the amount of test substance normally applied could exceed the highly toxic dose and, consequently result in the death or severe suffering of the animals. In addition, when dermal toxicity studies utilizing albino rabbits have already been performed up to the limit dose level of 2000 mg/kg body weight or higher, and no dermal irritation or corrosion has been seen, additional testing for skin irritation/corrosion may not be needed. A number of considerations should be borne in mind when evaluating acute dermal toxicity in previously performed studies.
For example, reported information on dermal lesions may be incomplete. Testing and observations may have been made on a species other than the rabbit, and species may differ widely in sensitivity of their responses. Also the form of test substance applied to animals may not have been suitable for assessment of skin irritation/corrosion (e.g. dilution of substances for testing dermal toxicity**.** However, in those cases in which well-designed and conducted dermal toxicity studies have been performed in rabbits, negative findings may be considered sufficient evidence that the substance is not corrosive or irritating.

Step 5,6 - Results from *in vitro* or *ex vivo* tests for skin corrosion and skin irritation. Substances that have demonstrated corrosive or severe irritant properties in a validated and accepted *in vitro* or *ex vivo* test designed for the assessment of these specific effects, need not be tested in animals. It can be presumed that such substances will produce similar severe effects *in vivo*.

Step 7,8 - *In vivo* test in rabbits. Should a weight-of the-evidence decision be made to conduct *in vivo* testing, it should begin with an initial test using one animal. If the results of this test indicate the substance to be corrosive to the skin, further testing should not be performed. If a corrosive effect is not observed in the initial test, the irritant or negative response should be confirmed using up to two additional animals for an exposure period of four hours. If an irritant effect is observed in the initial test, the confirmatory test may be conducted in a sequential manner, or by exposing the two additional animals simultaneously.

Figure A11.1 *Testing strategy for skin corrosion and irritation testing as appended to OECD TG 404 (5) on dermal corrosion and irritation.*

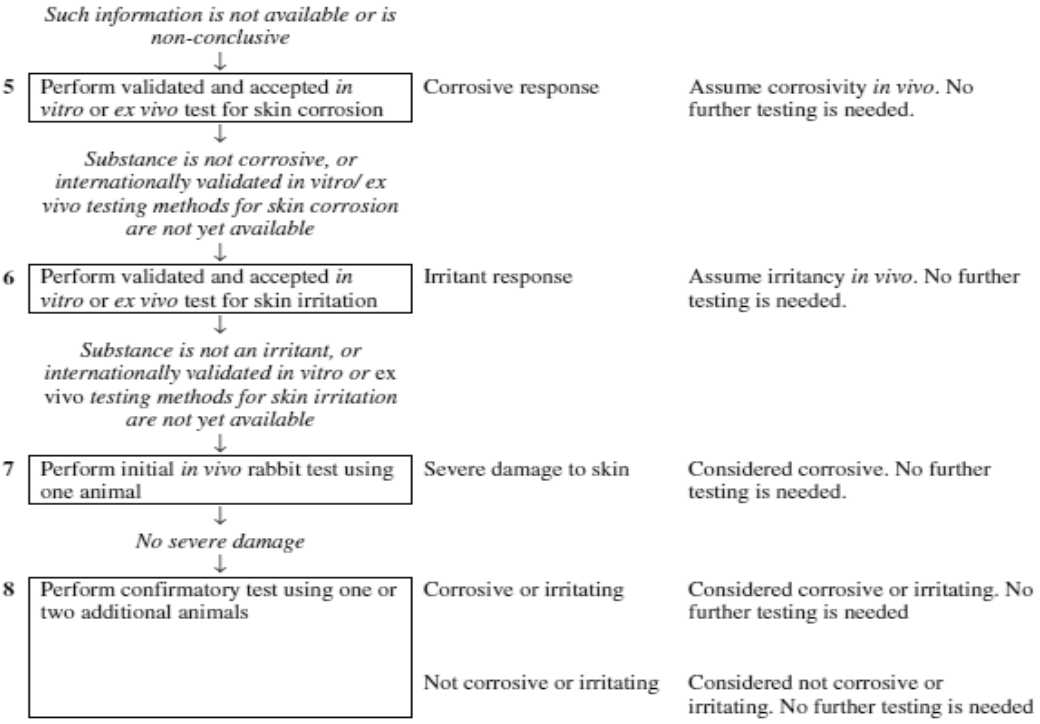
TESTING AND EVALUATION STRATEGY FOR DERMAL IRRITATION /CORROSION

	Activity	Finding	Conclusion
1	Existing human and/or animal data showing effects on skin or mucous membranes	Corrosive	Apical endpoint; considered corrosive. No testing is needed.
		Irritating	Apical endpoint; considered to be an irritant. No testing is needed.
		Not corrosive/not irritating	Apical endpoint; considered not corrosive or irritating. No testing is needed.
	↓ *No information available, or available information is not conclusive* ↓		
2	Perform SAR evaluation for skin corrosion/irritation	Predict severe damage to skin	Considered corrosive. No testing is needed.
		Predict irritation to skin	Considered an irritant. No testing is needed.
	↓ *No predictions can be made, or predictions are not conclusive or negative* ↓		
3	Measure pH (consider buffering capacity, if relevant)	pH ≤ 2 or ≥ 11.5 (with high buffering capacity, if relevant)	Assume corrosivity. No testing is needed.
	↓ *2<pH < 11.5, or pH ≤ 2.0 or ≥ 11.5 with low/no buffering capacity, if relevant* ↓		
4	Evaluate systemic toxicity data via dermal route [1]	Highly toxic	No further testing is needed.
		Not corrosive or irritating when tested to limit dose of 2000 mg/kg body weight or higher, using rabbits	Assume not corrosive or irritating. No further testing is needed.
	↓		

[1] Can be considered before Steps 2 and 3.

Figure A11.1 continued on next page

Figure A11.1 continued



5. Application of the TG 404 Test Strategy to the substances listed by ICCVAM/NICEATM

Table A11.1 lists those corrosive substances identified by NICEATM/ICCVAM that may be incorrectly predicted as negatives ("non-corrosives") by the some of the validated skin corrosion tests. The substances are numbered alphanumerically from A to O and represent various categories of chemicals such as *inter alia* amines, organic saturated acids carrying carboxyl residues, metal salts etc.

Table A11.2 shows the step-wise application of the testing strategy on these chemicals and the resulting conclusions. Note that for the sake of simplicity table A11.2 does not show acute dermal toxicity testing data as required for step 4 of the strategy. These data are separately listed in table A11.3

Table A11.1 *List of chemicals identified by NICEATM/ICCVAM as potentially under-predicted by the in vitro skin corrosion tests (6)*

	Chemical	**CAS No.**	**Chemical Class** ***(8)***	**EU Class** ***(8)*** R34-corrosive R35-severly corrosive	**UN Group** ***(8)***	**Physical State** ***(8)***
A	1-(2-Aminoethyl) piperazine	140-31-8	organic base	R34	II	Liquid
B	n-Heptylamine		organic base	R34	II/III	Liquid
C	Dimethyldipropylenetriamine	10563-29-8	inorganic base	R35	I	Liquid
D	Phosphorus tribromide	7789-60-8	inorganic acid	R35	I	Liquid
E	Allyl bromide	106-95-6	electrophile	R34	II/III	Solid
F	Caprylic acid (synonyme octanoic acid)	124-07-02	organic acid	R34	II/III	Liquid
G	Octanoic/decanoic acids (60:40)	68937-75-7	organic acid	R34	II/III	Liquid
H	Carvacrol	499-75-2	phenol	R34	II/III	Liquid
I	Iron III Chloride	7705-08-0	inorganic salt	R34	II	Solid
J	Methacrolein	78-85-3	electrophile	R34	II/III	Liquid
K	Sulfuric acid (15%) - in the skin corrosion validation study, 10% sulfuric was tested	7664-93-9	inorganic acid	R34/R35 (likely R34)	NA**	Liquid
L	Tallow amine	61790-33-8	organic base	R35	II	Wax/Tallow
	2-tert-Butylphenol	88-18-6	phenol	R34	II/III	Liquid
N	2-Mercapoethanol, sodium salt **(45% aq.)**	37482-11-4	inorganic base	R34	II/III	Liquid
O	Glycol bromoacetate **(85%)**	3785-34-0	electrophile	R34	II/III	Liquid

Table A11.2 *Application of the testing strategy of TG 404 to the chemicals listed by NICEATM/ICCVAM (table A11.1)*
Note: Data on dermal toxicity LD-50 (Step 4) not included in this table but shown in table A11.3. C – Corrosive, NC-Non corrosive, R38 – EU skin irritant. Grey shadowing identifies step in which chemical was classified as corrosive. With respect to the in vitro skin corrosivity test methods, three validated assays (EPISKIN, EpiDerm and SkinEthic) are listed which are based on the same test system as the corresponding skin irritation assays described in this background document. Data on sulphuric acid and octanoic acid are moreover available from the validated EST-1000 method (ESAC statement 2009). Both substances are correctly predicted as corrosives by the EST-1000 assay[4] .

		STEP 1 – Available human and animal data from controlled 4h HPT	**STEP 2 QSAR C-corrosive, NC-non corrosive**		**STEP 3 pH, reactivity, volatility and other physico-chemical properties and observations**			**STEP 5 Skin corrosion tests using 3 validated in vitro skin corrosion RhE methods**				**STEP 6 Skin irritation results - validated in vitro skin irritation test method – EpiDerm model**
	Chemical		**SAR - analysis (1)**	**Comment on SAR analysis** (8)	**pH measurement, pH<2 and >11.5 classifies the chemicals as C (8)**	**Comments from Barrat et al. 1998 (8)**	**MTT interaction (MTT reduction) Observations from skin corrosion testing**	**Corrosion EPISKIN (9) - killed controls not performed**	**Corrosion EpiDerm (10) - Phase II and Phase III) - killed controls not performed**	**Comments on corrosion data of EPISKIN and EPIDERM (11)**	**Corrosion SkinEthic – (12)**	**Iritation EpiDerm –(7) killed controls not performed**

[4] **J. Hoffmann, E. Heisler, S. Karpinski, J. Losse, D. Thomas, W. Siefken, H.-J. Ahr, H.-W. Vohr and H.W. Fuchs.** Epidermal-skin-test 1000 (EST-1000)—A new reconstructed epidermis for in vitro skin corrosivity testing. Toxicology in Vitro, Volume 19, Issue 7, 925-929.

A	**1-(2-Aminoethyl) piperazine**		C		8		YES	1C/8NC	C			positive
B	**n-Heptylamine**		C		8.4		YES	9 NC	4 C/ 2NC	result can be corrected completely by use of killed controls and reference filter	C-after correction with killed controls)	positive
C	**Dimethyldipropylenetriamine**		C		8.3		YES	9 C	8 C/1 NC	result can be corrected completely by use of killed controls and reference filter		positive
D	**Phosphorus tribromide**		Data not available		**1**	Highly volatile, decompose		9 C	6 C			positive
E	**Allyl bromide**		C		3.9 (for 10% solution)			9 C	C			
F	**Caprylic acid (Octanoic acid)**	Human irritant only - positive in 48/63 of human volunteers (13)	C	**Borderline** C - NC chemical as judged subjectively from the proximity of the chemical to the classification boundary (SAR	3.6			9 C	6 C		9 C	positive

				analysis)								
G	**Octanoic/decanoic acids (60:40)**	Decanoic acid is classified as human irritant in the 4h HPT (13, 14)	Data not available		3.9			9 C	6 C			
H	**Carvacrol**		NC	**Borderline** C - NC chemical as judged subjectively from the proximity of the chemical to the classification boundary (SAR analysis)	3.9		YES	9 C	NC	result can be corrected completely by use of killed controls and reference filter		positive
I	**Iron III Chloride**		Data not available		1 (for 10% solution)	Colored test material		5C / 4NC	C/NC	this chemical affect the edge of the insert and passes extremely quickly into the media that has buffering effect. Therefore it is sometimes undepredicted		positive

J	**Methacrolein**		C		3.6	Reducing agent (may affect MTT assay)	YES	6C/3NC	5 NC/1 C	result can be corrected completely by use of killed controls and reference filter		positive
K	**Sulfuric acid** (15%) - in the skin corrosion study, 10% was tested	10% caused no Effects in Human (15) 5-15% is labeled as R38 in EU	Data not available		1.2 (for 10%)			9 C (for 10%)	C (for 10%)		NC/C** (for 10%)	positive
L	**Tallow amine - not readily available**	The in vivo exposure time may have been greater than 3 min. Necrosis was observed in two of the three rabbits only from day 7. (8)	C	**Borderline** C - NC as judged from the QSAR analysis	ND		YES	9 NC				Chemical is not readily available
M	**2-tert-Butylphenol**		NC	**Borderline** C - NC chemical as judged subjectively from the proximity of	3.9			9 C	5C/1NC	result can be corrected completely by use of killed controls and reference filter	9 C	positive

				the chemical to the classification boundary (SAR analysis)								
N	**2-Mercapoethanol, sodium salt (45% aq.)** - not comercially available		Data not available		12	Reducing agent (may affect MTT assay)		9 NC		result can be likely corrected by use of killed controls and reference filter		Chemical is not commercially available
O	**Glycol bromoacetate (85%)** - not comercially available		Data not available		2		?	9 C				Chemical is not commercially available

Table A11.3 *LD 50 values (dermal route) for the chemicals listed by NICEATM/ICCVAM (see table A11.1).*

	Chemical	CAS No.	LD50 dermal (mg/kg)	animal	source	Web page
A	1-(2-Aminoethyl) piperazine	140-31-8	866.8	rabbit	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&resultsN4=140-31-8&N4=140-31-8&D7=0&D10=140-31-8&N25=0&N1=S_ID&ST=RS&F=PR&N3=mode%2Bmatchpartialmax&locale=en_US&countryCode=SK8&N25=0&N1=S_ID&ST=RS&F=PR&N3=mode%2Bmatchpartialmax&locale=en_US&countryCode=SK
			880.0	rabbit	MSDS Hansa Group	www.hansa-group.com/download=products/safetysheet/EN-E002010.pdf
B	n-Heptylamine	111-68-2	Not found		MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=n-Heptylamine&D7=0&D10=n-Heptylamine&N1=S_ID&ST=RS&N25=0&F=PR
C	Dimethyldipropylenetriamine	10563-29-8	1 310.0	rat	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&resultsN4=10563-29-8&N4=10563-29-8&D7=0&D10=10563-29-8&N25=0&N1=S_ID&ST=RS&F=PR&N3=mode%2Bmatchpartialmax&locale=en_US&countryCode=SK
			1 310.0	rat	MSDS Arkema Inc.	http://arkemaus.com/msds/1459.pdf
D	Phosphorus tribromide	7789-60-8	Not found		MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=7789-60-8&D7=0&D10=7789-60-8&N1=S_ID&ST=RS&N25=0&F=PR

E	Allyl bromide	106-95-6	Not found		MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=106-95-6&D7=0&D10=106-95-6&N1=S_ID&ST=RS&N25=0&F=PR
F	Caprylic acid	124-07-2	> 5 000	rabbit	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=124-07-2&D7=0&D10=124-07-2&N1=S_ID&ST=RS&N25=0&F=PR
			> 5 000	rabbit	MSDS Parchem	http://www.parchem.com/siteimages/Attachment/Caprylic%20Acid%20MSDS.pdf
G	octanoic/decanoic acids (60:40)	68937-75-7	Not found	Not found	Not found	Not found
H	carvacrol	499-75-2	2 700.0	rabbit	Internet link	http://www.thegoodscentscompany.com/data/rw1027311.html
			680.0*	mouse	Internet link	http://www.thegoodscentscompany.com/data/rw1027311.html
I	Iron III chloride	7705-08-0	Not found	rabbit	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=7705-08-0&D7=0&D10=7705-08-0&N1=S_ID&ST=RS&N25=0&F=PR
			Not found		MSDS ScholAR Chemitry	http://www.sargentwelch.com/pdf/msds/Iron_III_Chloride_Anhydrous_374.00.pdf
J	Methacrolein	78-85-3	366.0	rabbit	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&N3=mode+matchpartialmax&N4=78-85-3&D7=0&D10=78-85-3&N1=S_ID&ST=RS&N25=0&F=PR
			430.0 (µl/kg)	rabbit	MSDS acc. to OSHA and ANSI	http://www.labseeker.com/ChemicalBiotech/msds%5CAlfa%5C43000%5C43124.pdf

K	Sulfuric acid (15%) - in the skin corrocion validation study 10% sulfuric acid was tested	7664-93-9	Not found		SIDS Initial Assessment Report for 11th SIAM, sulfuric acid MSDS AppliChem	http://www.chem.unep.ch/irptc/sids/OECDSIDS/7664939.pdf http://www.applichem.de/fileadmin/datenblaetter/a1446_en.pdf
L	Tallow amine	61790-33-8	Not found	Not found	Not found	Not found
M	2-tert-Butylphenol	88-18-6	705.0	rat	MSDS Sigma Aldrich	http://www.sigmaaldrich.com/catalog/Lookup.do?N5=All&resultsN4=88-18-6&N4=88-18-6&D7=0&D10=88-18-6&N25=0&N1=S_ID&ST=RS&F=PR&N3=mode%2Bmatchpartialmax&locale=en_US&countryCode=SK#test
N	2-Mercapoethanol, sodium salt (45 % aq.)	37482-11-4	Not found	Not found	Not found	Not found
O	Glycol bromoacetate (85 %)	3785-34-0	Not found	Not found	Not found	Not found

*) subcutaneous

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