



Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A tiered approach to the use of alternatives to animal testing for the safety assessment of cosmetics: Skin irritation

Martin Macfarlane^a, Penny Jones^a, Carsten Goebel^b, Eric Dufour^c, Joanna Rowland^d, Daisuke Araki^e, Margit Costabel-Farkas^f, Nicola J. Hewitt^g, Jalila Hibatallah^h, Annette Kirstⁱ, Pauline McNamee^j, Florian Schellau^{f,k}, Julia Scheel^{l,*}

^aUnilever, Colworth Science Park, Sharnbrook, Bedford MK44 1LQ, UK

^bProcter & Gamble Darmstadt Innovation Center, Berliner Allee 65, 64274 Darmstadt, Germany

^cL'Oreal, River Plaza 25-29, quai Aulagnier 92600 Asnières-sur-Seine, France

^dGlaxoSmithKline Consumer HealthCare R&D, Weybridge, Surrey KT13 ODE, UK

^eKanebo Cosmetics, 89/91 Rue du Faubourg St Honoré, 75008 Paris, France

^fJohnson & Johnson GmbH, Kaiserswerther Straße 270, 40474 Düsseldorf, Germany

^gScientific Writing Services, Wingertstrasse 25, 64390 Erzhäusen, Germany

^hChanel Parfums Beauté 135, Avenue Charles de Gaulle, 92525 Neuilly sur Seine, France

ⁱKPSS – Kao Professional Salon Services GmbH, Pfungstaedter Strasse 92-100, 64297 Darmstadt, Germany

^jProcter & Gamble, Rusham Park Technical Centre, Whitehall Lane, Egham, Surrey, TW20 9NW, UK

^kColipa, Avenue Herrmann Debroux 15A, B-1160 Auderghem, Brussels, Belgium

^lHenkel AG & Co. KGaA, Henkelstraße 67, 40191 Düsseldorf, Germany

ARTICLE INFO

Article history:

Received 23 January 2009

Available online 22 April 2009

Keywords:

Skin irritation

Safety assessment

Alternative methods

ABSTRACT

Evaluation of the skin irritancy and corrosivity potential of an ingredient is a necessity in the safety assessment of cosmetic ingredients. To date, there are two formally validated alternatives to the rabbit Draize test for skin corrosivity in place, namely the rat skin transcutaneous electrical resistance (TER) assay and the Human Skin Model Test using EpiSkin™, EpiDerm™ and SkinEthic™ reconstructed human epidermal equivalents. For skin irritation, EpiSkin™, EpiDerm™ and SkinEthic™ are validated as stand-alone test replacements for the rabbit Draize test. Data from these tests are rarely considered in isolation and are evaluated in combination with other factors to establish the overall irritating or corrosive potential of an ingredient. In light of the deadlines established in the Cosmetics Directive for cessation of animal testing for cosmetic ingredients, a COLIPA scientific meeting was held in Brussels on 30th January, 2008 to review the use of alternative approaches and to set up a decision tree approach for their integration into tiered testing strategies for hazard and safety assessment of cosmetic ingredients and their use in products. In conclusion, the safety assessments for skin irritation/corrosion of new chemicals for use in cosmetics can be confidently accomplished using exclusively alternative methods.

© 2009 Elsevier Inc. All rights reserved.

* Corresponding author. Fax: +49 211 798 12413.

E-mail address: julia.scheel@henkel.com (J. Scheel).

¹ Abbreviations used: SCCP, Scientific Committee on Consumer Products; BfR, Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment, Germany); COLIPA, The European Cosmetic Association; DEREK, Deductive Estimation of Risk from Existing Knowledge; DSS, Decision Support System; ECVAM, European Centre for the Validation of Alternative Methods; ESAC, ECVAM Scientific Advisory Committee; GHS, globally harmonised System; IL-1 α , interleukin-1 α ; IL-8, interleukin-8; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium salt; NR, neutral red; OECD, Organisation for Economic Co-operation and Development; (Q)SAR, (Quantitative) Structure Activity Relationship; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; RHE, reconstructed human epidermis; SICRET, Skin Irritation Corrosion Rules Estimation Tool; SIT, skin irritation test; TG, Test Guideline; TTC, Threshold of Toxicological Concern; WoE, Weight of Evidence.

1. Introduction

Skin irritation is defined as reversible damage of the skin following the application of a test substance for up to 4 h (Scientific Committee on Consumer Products (SCCP, 2006)).¹ By contrast, skin corrosion is defined as “irreversible damage to the skin, namely visible necrosis through the epidermis and into the dermis, following the application of a test substance for the duration period of 3 min up to 4 h (Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 404). The potential of chemicals to cause acute skin irritation must be assessed as part of a basic toxicology program. Historically, the evaluation of the potential of a chemical to produce skin irritation has been carried out using the Draize skin irritation test in rabbits according to OECD TG 404 and Method B.4 of Annex V to Directive 67/548/EEC (OECD TG 404, 2002; EU, 1967)

(Directive 67/548/EEC is now being replaced by regulations 1272/2008/EC (“EU–GHS” (Globally Harmonised System)) and 440/2008/EC on test methods (EU, 2008a,b)). The chemical is applied to the shaved area of skin and the appearance of oedema and/or erythema is determined up to 72 h after application. Despite the universal acceptance of this assay, the correlation between animal and human irritancy has come under question since there have been some cases where chemicals have been misclassified using *in vivo* rabbit data (Basketter et al., 1999; York et al., 1996). The main reason for the discrepancy is thought to be related to a higher susceptibility of the rabbit to skin reactions, possibly due to differences in skin structure between humans and rabbits.

The SCCP is the committee which provides the European Commission with scientific opinions on the safety of non-food consumer products, including cosmetics and therefore, alternative methods must also meet their acceptance criteria. The European Centre for the Validation of Alternative Methods (ECVAM), which is independent from the SCCP, was set up to ensure that alternative methods meet both scientific and regulatory approval. The acceptance of the methods depends on their validation as well as their long-term reproducibility and consistency (Rispin et al., 2004). A number of alternative assays have been developed to determine the skin irritation potential of chemicals but the most significant milestone was the recent acceptance by the ECVAM Scientific Advisory Committee (ESAC) of the use of the EpiSkin™ reconstructed human epidermis (RHE), EpiDerm™ skin irritation test (SIT) and SkinEthic™ RHE *in vitro* skin models as validated stand-alone replacements for the rabbit Draize test, distinguishing between skin irritating (R38, similar to GHS Category 2) and non-irritating (no-label) chemicals (ESAC, 2008).

On 11 March 2009, two bans entered into force concerning animal testing related to cosmetics products in the European Union. Both were decided in 2003 in the context of the 7th amendment to the Cosmetics Directive (EU, 1976), which, amongst other purposes, aims at ensuring the safety of ingredients used in cosmetic products. A first ban concerns animal testing itself to assess the safety of ingredients. A second ban prohibits the sale of cosmetic products containing ingredients tested on animals. This ban is progressive, until it becomes a complete ban in March 2013 taking into account scientific progress being made regarding repeat dose tests for which alternative methods do not yet exist. The impact of the ban on the use of alternative assays to replace animal tests for the assessment of skin irritation after March 2009 was analysed at a COLIPA (The European Cosmetics Association) scientific meeting organised by its Safety Assessment and Skin Tolerance Project Teams in Brussels on 29th January, 2008. Participants included representatives from a number of cosmetic companies. Decision tree approaches to safety assessment of chemicals used in

cosmetics were developed based on the discussions held during the workshop.

2. Results and discussion

2.1. Current alternative approaches to the assessment of skin irritation

Current safety assessment practice makes routine use of integrated testing strategies based on a Weight of Evidence (WoE) approach. WoE approaches have been in use for long and have also been investigated by ECVAM in the context of validation (Balls et al., 2006). The principle is that all available information is considered in the assessment, in this case of skin irritation. Such information may include, for example:

- Physicochemical properties.
- *In silico* methods.
- Historical *in vivo* animal data (including dermal toxicity data, data from skin sensitisation studies).
- *In vitro* data.
- Human data (clinical and post-market surveillance/history of safe use).
- Exposure.

2.2. Alternative *in vitro* approaches already applicable for safety assessment

Tables 1 and 2 list some of the alternative skin models to identify skin corrosives (Table 1) and irritants (Table 2), together with a brief description of the assays and the information that is obtained from them. *In vitro* methods for the assessment of corrosivity are already accepted and formally established as OECD guidelines for long and data from such assays have clearly their role in the testing strategy for skin irritation. Since the main focus of our investigation is the assessment of skin irritation, we concentrate here on assays for the assessment of skin irritation. Keratinocytes play an important role in the initiation, modulation and regulation of skin irritation (Coquette et al., 2000), therefore, they have been central to the development of *in vitro* models to evaluate this effect. In contrast to some of the monolayer cell models, RHE models have a functional stratum corneum and can therefore be used to test neat (hydrophilic and hydrophobic) chemicals added directly to the stratum corneum. One main difference between the RHE models and normal human skin is the rate of penetration of chemical into the skin, which are higher in RHE skin equivalents than in human epidermis (Schäfer-Korting et al., 2008). This difference gives rise to a higher sensitivity of the RHE models, however, this can be considered to be an advantage when testing mild irritants (Perkins

Table 1
Overview of alternative skin corrosion assays and their application.

Assay	Evaluation end point	Chemical class applicability based on current practices and literature	Reference ^a
Rat skin transcutaneous electrical resistance (TER) test	Barrier function. Reduction in the TER	Identifies corrosives and non-corrosives. Solids, liquids, and emulsions of any chemical or product class	Oliver et al. (1986), Fentem et al. (1998), Grindon et al. (2007)
<i>In vitro</i> reconstituted human epidermal (RHE) skin corrosion models e.g., EpiSkin™, EpiDerm™, SkinEthic™, EST-1000™	MTT metabolism is measured as a marker of skin viability	Identifies corrosives and non-corrosives. Avoid chemicals that react with the trans-well plastic plates	Kaufmann et al. (2000), Faller et al. (2002), Hoffman et al. (2005); www.cellsystems.de; Spielmann et al. (2007), Grindon et al. (2007)
Reconstituted membrane barrier test: the Corrositex™ membrane barrier test	Penetration of test substances through a hydrogenated collagen matrix (biobarrier) causing a colour change in the buffer below	Identifies corrosives and non-corrosives. Overall predictivity of this model is low and the number of chemicals used in cosmetics which are qualified for testing using this assay are limited	Stobbe et al. (2003), Grindon et al. (2007)

^a Original references and reviews of different models for skin corrosion are cited.

Table 2
Overview of alternative skin irritation assays and their application.

Assay	Evaluation end points which can be measured	Applicability based on current practices and literature ^a		Reference ^b
		Irritancy range detected ^a	Chemical class	
EpiSkin™ model	Viability measured by MTT metabolism, cytokine release e.g., IL-1 α	Irritants and non-irritants	A diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants Fentem et al. (1998)	Williams and Kupper (1996), Spielmann et al. (2007), Coquette et al. (2003, 2005), Welss et al. (2004); www.invitroskin.com
EpiDerm™ model	Viability measured by MTT metabolism, cytokine release e.g., IL-1 α	Irritants and non-irritants	A diverse group of chemicals with varying physicochemical properties	Faller et al. (2002), Spielmann et al. (2007); www.mattek.com
SkinEthnic™ RHE model	Viability measured by MTT metabolism, cytokine release e.g., IL-1 α , tissue histology	Irritants and non-irritants. Including IL-8 can help to discriminate between irritant and sensitizing chemicals	A diverse group of chemicals with varying physicochemical properties	Kandárová et al. (2004, 2006), Tornier et al. (2006), Coquette et al. (2003); www.skinethic.com
Prediskin	Viability measured by MTT metabolism, tissue histology	Irritants and non-irritants	Not defined due to low predictivity	www.biopredic.com
Mouse skin integrity function test (SIFT) (ex-vivo)	Trans-epidermal water loss, electrical resistance	Low predictive power	Not defined due to low predictivity	Heylings et al. (2001, 2003), Spielmann et al. (2007)
Cultured keratinocytes	Release of cytokines e.g., IL-1 α and TNF- α	Irritants and non-irritants. The lack of a stratum corneum may result in high sensitivity and over-predict the irritation potential	Avoid poorly water-soluble chemicals	Spiekstra et al. (2005), DeLeo et al. (1996), Gueniche and Ponec (1993), Müller-Decker et al. (1994), Jacobs et al. (1989), van de Sandt et al. (1999)
Cultured human keratinocytes or mouse embryo fibroblasts 3T3 cells	Viability measured by NR uptake, morphology, expression and release of cytokines, skin physiology	Irritants and non-irritants. The lack of a stratum corneum may result in high sensitivity and over-predict the irritation potential	Avoid poorly water-soluble chemicals	Sanchez et al. (2006), Benavides et al. (2004), Lee et al. (2000)

^a Irritancy range and wording based on original references and does not include ECVAM and regulatory publications.

^b Original references are cited.

et al., 1999). Unlike the *in vitro* corrosion RHE assays, the skin irritation RHE assays may include additional endpoints such as cytokine release e.g., IL-1 α , a primary event in inflammation (Williams and Kupper, 1996; Spielmann et al., 2007; Coquette et al., 2003, 2005; Welss et al., 2004). Some protocols use short incubation times of 4 h to mimic the *in vivo* human patch test (Tornier et al., 2006), whereas others use longer times of 16 h (Faller et al., 2002) and 42 h (Spielmann et al., 2007) which allow recovery from weak effects and advancement of significant effects. There are RHE models which have been developed using different combinations of endpoints, namely EpiSkin™, EpiDerm™, SkinEthic™, Prediskin™, LSE/HSE, Re-DED, Aligraf and Skin2 reviewed by Welss et al. (2004), some of which are shown in Table 2.

Table 3 outlines the regulatory status of *in vitro* skin irritation assays to detect corrosive and irritant chemicals. There are a number of validated *in vitro* assays to predict skin corrosivity and, to a lesser extent, skin irritation. Whilst the models listed are mainly used or validated for classification and labelling purposes, they can also be used as part of a safety assessment/benchmarking approach in consumer and occupational safety assessment. Currently, the ESAC fully endorses three *in vitro* skin irritation models, namely EpiSkin™, EpiDerm™ and SkinEthic™ as stand-alone replacements for the Draize skin irritation test in animals. Moreover, these tests can be used as stand-alone tests to distinguish between skin irritating and non-irritating chemicals. The current SCCP statement (SCCP, 2007) welcomes the use of the EpiSkin™ model but still expresses concerns about the relevance of this test to assess the skin irritation potential of cosmetics. The concerns raised were twofold:

- The group of chemicals used to validate the assay, although included a large number of cosmetic ingredients, only contained one of the reference compounds listed in the Annexes to the

Directive 76/768/EEC. The cosmetic industry has addressed this concern by submitting EpiSkin™ data on over 20 cosmetic ingredients included in the Annexes of the Cosmetics Directive. This dataset conclusively shows that regulated cosmetic ingredients belong to the application domain of the EpiSkin™ test method, and that predictive performance of this test is similar for such ingredients and chemicals in general.

- Colouring chemicals that may interfere with the read-out system (MTT assay) need to have additional controls in order to take into account the non-specific optical density due to the residual test substance colour. An industry report addressing this issue has been submitted and is currently under review by the SCCP/SCCS. It shows that such interferences are overall infrequent and that when they occur, testing appropriate dilutions of the colouring materials permit to reduce this unspecific colouration to acceptable levels and to evaluate their skin irritation potential.

2.3. Alternative approaches not yet validated and/or under development

2.3.1. *In vitro* approaches

There are other *in vitro* skin irritation models which have not yet been formally validated or received regulatory acceptance but have proved useful in assessing the local tolerance of cosmetic ingredients using a WoE approach. Some of these are listed in Table 2, however, it is recognised that this list is not exhaustive. There are other endpoints which are considered useful, such as IL-8 levels, lactate dehydrogenase release and histological analysis (Welss et al., 2004).

2.3.2. *In silico* approaches

Computational models are available for Quantitative Structure Activity Relationships ((Q)SAR), incorporating physicochemical

Table 3
Regulatory status of alternative skin corrosion and irritation assays.

Assay	Validation organisation acceptance	Regulatory acceptance	Applicability as referenced by authorities	On-going development to promote external acceptance
<i>Skin corrosion</i>				
Rat skin transcutaneous electrical resistance (TER) test	ECVAM validation study in 1996–1997 Fentem et al. (1998). ESAC recommended that this assay be used as a stand-alone replacement for animal studies to identify corrosive and non-corrosive chemicals	Accepted by EU and US for the prediction of the skin corrosion potential OECD TG 430 (2004)	Corrosive and non-corrosive chemicals. Not used to determine degree (i.e., potency) of corrosives	Protocol currently being optimized following ECVAM pre-validation study to reduce FPs with surfactants and neutral organics
<i>In vitro</i> reconstituted human epidermal (RHE) skin corrosion models: EpiSkin™, EpiDerm™, EST1000 and SkinEthic™	RHE models are accepted by ESAC as validated assays to replace animal skin corrosion studies	Regulatory accepted for the prediction of the skin corrosion potential, using MTT as the end point (OECD protocol number 431, ESAC statement 21, March 2000)	Corrosive and non-corrosive chemicals	
Epidermal skin test (EST1000) model	Recognised in the scientific literature to have a good <i>in vitro:in vivo</i> correlation, using the twelve reference compounds stipulated by the OECD TG 431 corrosivity protocol	Not accepted by regulatory authorities for identifying skin irritants	Corrosive and non-corrosive chemicals	Needs a “catch-up validation” in order to match the supporting data available for the other models
Reconstituted membrane barrier test: the Corrositest™ membrane barrier test	The Corrositest™ model has been endorsed by ESAC and adopted by the OECD	Regulatory accepted for the prediction of the skin corrosion potential OECD TG 435, currently under new draft discussions	Corrositest™ used as part of a tiered strategy but only for the determination of the corrosive potential of specific chemicals which cause a change in pH, such as organic bases and inorganic acids ESAC, (2000)	
<i>Skin irritation</i>				
EpiSkin™	In April, 2007, this model (using MTT reduction and IL1a release) was passed by ECVAM as a validated alternative to the <i>in vitro</i> irritation test	Accepted by EU for the prediction of skin irritation potential	R38 (label for skin irritation) and non-irritating (no label) chemicals	The cosmetic industry submitting EpiSkin™ data on over 20 cosmetic ingredients included in the Annexes of the Cosmetics Directive. Experimental findings on the effect of coloured chemicals on the outcome of this model are to be published
EpiDerm™	In November, 2008, EpiDerm SIT (using MTT reduction) was passed by ECVAM as a validated alternative to the <i>in vitro</i> irritation test, following update validation based on a modified protocol of the validated EpiDerm model (validated as part of a testing strategy)	Accepted by EU for the prediction of skin irritation potential	R38 (label for skin irritation) and non-irritating (no label) chemicals	
SkinEthic™ RHE model	In November, 2008, this model (using MTT reduction) was passed by ECVAM as a validated alternative to the <i>in vitro</i> irritation test	Accepted by EU for the prediction of skin irritation potential	R38 (label for skin irritation) and non-irritating (no label) chemicals	
Epidermal skin test (EST1000) model		Not accepted by regulatory authorities for identifying skin irritants		Needs a “catch-up validation” in order to match the supporting data available for the other models
PrediSkin	ECVAM pre-validation study concluded that this method was not ready to enter a formal validation study. Protocol was over-sensitive, resulting in the prediction of all the non-irritant chemicals as irritants Fentem et al. (2001)	Not accepted by regulatory authorities for identifying skin irritants		
Mouse skin integrity function test (SIFT) (<i>ex-vivo</i>)	Dropped from ECVAM validation study due to low predictive power Spielmann et al. (2007)	Not accepted by regulatory authorities for identifying skin irritants		Heylings et al. (2001, 2003), Spielmann et al. (2007)

and other characteristics to predict the irritation potential of chemicals (http://ecb.jrc.ec.europa.eu/documents/QSAR/QSAR_Review_Irritation.pdf). (Q)SARs are theoretical models that predict the “approximate relationship between a biological property of a compound and its structure-derived physicochemical and structural properties” (Johnson and Maggiora, 1990). A decision support

system (DSS) developed by the German Federal Institute for Risk Assessment (BfR) uses physicochemical exclusion rules to predict the absence of skin irritation/corrosion potential in combination with structural inclusion rules (SARs) to predict the presence of such potential (Gerner et al., 2004a,b). The exclusion rules are based on physicochemical properties such as molecular weight,

Table 4
In silico approaches used to predict skin irritation.

Application	Description	History
OECD (Q)SAR Application Toolbox	Incorporates information into a logical workflow by grouping chemicals into chemical categories (www.oecd.org). Evaluates the hazard based on the overall data set of the category, which must not have every chemical tested for every end point. Read-across from one tested chemical to an untested chemical is carried out to fill the data gaps	The first version was released in March 2008 and outlines the technological proof-of-concept. Evaluation by RIVM 2005 Rorije and Hulzebos (2005)
TOPKAT	A statistically based system consisting of a number of robust, cross-validated (Q)SAR models (www.accelrys.com) derived from large data sets of toxicological information from the literature. Chemicals are characterized according to structural, topologic, and electrotopologic indices. This system contains models based on data from 1258 compounds (www.accelrys.com) and can differentiate between irritants and non-irritants	A recent report demonstrated that TOPKAT performed well in predicting the skin irritation potential of the majority of a panel of 116 test chemicals Mombelli (2008)
DEREK	A knowledge based system comprising a number of structural rules (based on strongly acidic and basic features which relate to the parent molecules) that aim to encode structure-toxicity information with an emphasis on mechanisms	
Vitic database (Lhasa Ltd.)	A 'chemically intelligent' database which can recognise and search for similarities in chemical structures	
Decision support system (DSS) and (BfR)	The DSS SICRET (Skin Irritation Corrosion Rules Estimation Tool) model consists of a number of rules (known as the Gerner rules) based on physicochemical characteristics (such as melting point, logP and aqueous solubility) to exclude irritation, and structural alerts to include and predict corrosive chemicals (SICRET) and irritants Saliner et al. (2007)	This model correctly predicted 99.3% non-corrosives and 96.6% non-irritants Saliner et al. (2007)

aqueous solubility, and log Kow, whereas the inclusion rules are based on sub-structural molecular features. The physicochemical rules implicitly take into account bioavailability (skin penetration) whereas the structural rules take reactivity into account. The physicochemical and structural rulebases are designed to predict the EU risk phrases for skin irritation and skin corrosion. Further details are given in QSAR Reporting Format for the BfR skin and eye irrita-

tion rulebases. In one study focussing on esters ([Smith et al., 2000](#)), the best variables correlating to human skin irritation were water solubility (lower for irritants than non-irritants), a dispersion parameter (higher for irritants), a hydrogen-bonding parameter (higher for irritants), the sum of partial positive charges (lower for irritants), and density (lower for irritants). There are a number of *in silico* models available and these are outlined in [Table 4](#). Data

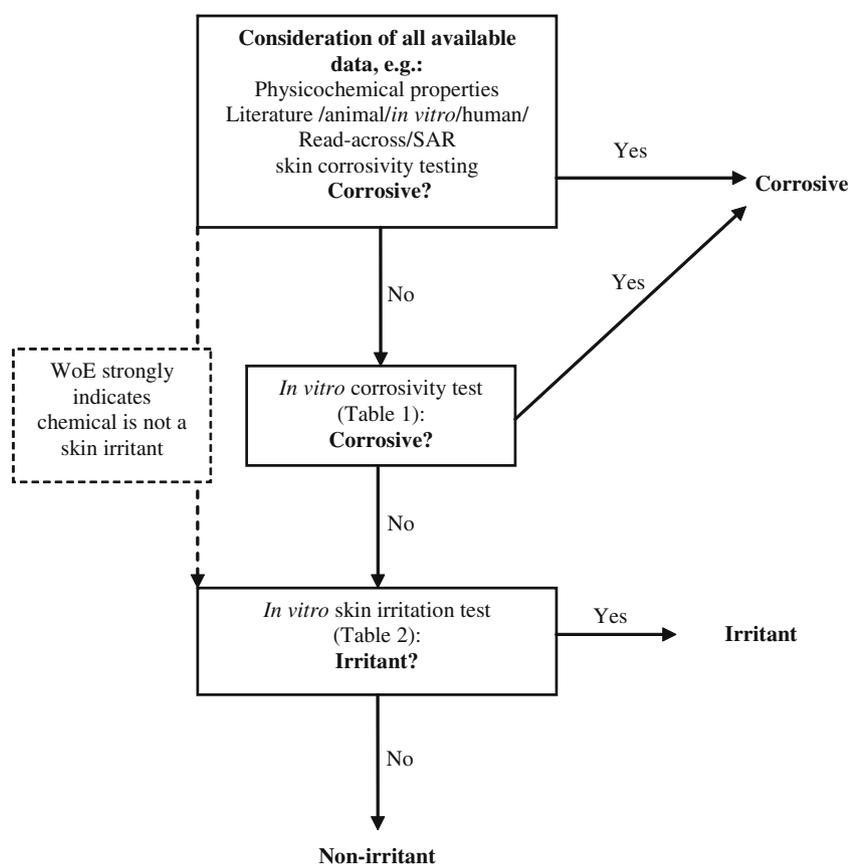
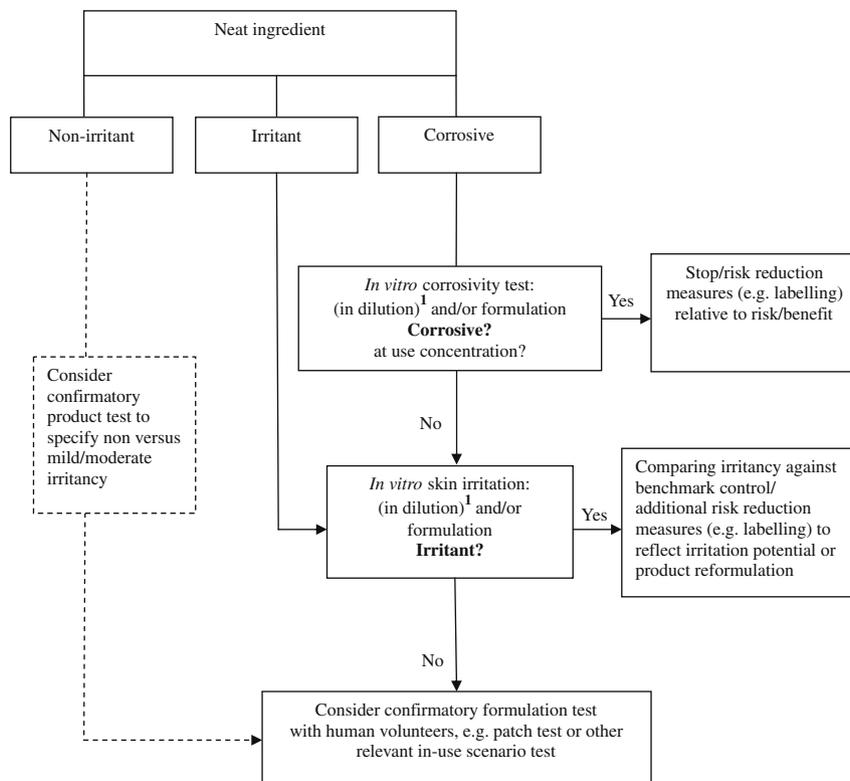


Fig. 1. Decision tree approaches to evaluate ingredients and final products for hazard assessment.



¹ *in vitro* tests to further position use concentrations of chemicals in dilutions are not currently accepted by authorities

Fig. 2. Decision tree approaches to evaluate ingredients and final products for safety assessment based on hazard data for the neat ingredient.

from *in vitro* methods can be used in combination with information on the physicochemical properties of the ingredient to provide information on its clinical tolerance. (Q)SARs might not constitute a full replacement by themselves, but they are valuable tools for screening and for prioritisation.

2.3.3. “Read-across” approach

The read-across approach uses the results obtained with a compound to predict the outcome of the effect of a chemically-related compound under evaluation. This may be based on chemical domain e.g., OECD Application Toolbox (www.oecd.org) or when animal data are available on similar substances. As with other end points (such as eye irritation) such evaluations are only possible when sufficient historical *in vivo* data for the chemical domain are available and there are no additional structural alerts that are considered likely to cause corrosive or irritant effects.

3. A tiered approach for skin irritation assessment

The OECD TG 404 recommends the use of a stepwise approach to evaluating the potential of a chemical to cause skin corrosion and/or irritation (OECD TG 404, 2002). A decision tree strategy for testing skin corrosion and irritation potential has been outlined recently by Grindon et al. (2007). Similarly, we have constructed two decision trees to aid approaches to using alternative models for hazard assessment (Fig. 1) and safety assessment (Fig. 2) of irritants. The decision trees can be used as a generic and broad overview of the type of questions needed to assess the potential hazard and the actual safety at usage levels. It should be kept in mind that it is not mandatory to carry out all the assays, rather they should be a guide as to what issues should be addressed, which assays could be used to resolve the issue and which extra assays could be used to add weight to the assessment.

3.1. Decision tree for hazard assessment

A decision tree for skin irritation hazard assessment is outlined in Fig. 1. It should be noted that decisions in this tiered approach take into account assays that already have regulatory acceptance as well as those that are described in the scientific literature as being suitable. This is a tiered approach using different steps:

Step 1: The initial step in hazard identification is based on the physicochemical properties (e.g., pH) and other available data (e.g., (Q)SAR human and/or animal data) of the undiluted chemical to answer the question “Does this chemical have the potential to be corrosive?”. A compound is considered corrosive if indicated by the physicochemical properties. For example, strong acids ($\text{pH} \leq 2$) or bases ($\text{pH} \geq 11.5$) are considered corrosive without the need for testing.

If the answer to this question is “yes”, the chemical is corrosive and labelled accordingly (R34, similar to GHS Category 1). If the answer to this question is “no”, proceed to Step 2. If the WoE strongly indicates that the undiluted chemical is also not a skin irritant, by-pass Step 2 and proceed to Step 3 (indicated by the dotted lines).

Step 2: Conduct an *in vitro* corrosivity test to answer the question “Is the corrosivity of the chemical confirmed?”. Appropriate assays accepted by regulatory authorities for the identification of *non-corrosive* chemicals and mixtures are shown in Table 3. If the outcome of this assay indicates that the chemical is corrosive, the labelling in Step 1 is confirmed. If the *in vitro* corrosivity test does not indicate corrosivity of the chemical, proceed to Step 3.

Step 3: Conduct an *in vitro* assay for skin irritation to answer the question “Is the chemical a skin irritant?”. Table 2 contains a list of assays that have been developed to evaluate skin irritation.

The only regulatory accepted stand-alone assay to determine the skin irritant potential of a chemical is the *in vitro* EpiSkin™ assay. If the outcome of assay(s) identifies the chemical as an irritant, it is labelled accordingly (irritant, R38, similar to GHS Category 2). If the skin irritation test is negative, then the chemical is non-irritating (no labelling/not classified (NC)).

3.2. Decision tree for safety assessment

A decision tree for safety assessment of chemicals using alternative skin irritation models is outlined in Fig. 2. At each step in safety assessment, a WoE approach (based on physicochemical properties and other available data) and read-across is recommended. Following the outcome of the hazard identification, there are three possible outcomes for safety assessment, namely, corrosive, irritant and non-irritating.

If the chemical is corrosive: A tiered approach using different steps is as follows:

Step 1: Conduct an *in vitro* corrosivity test using the relevant concentration of the chemical to answer the question “Is the chemical corrosive at the relevant concentrations?”.

If the answer to this question is “yes”, then the chemical is not further considered for cosmetic use or can be further tested in the intended formulation. If the outcome of the formulation test for corrosivity is negative then proceed to Step 2.

Step 2: Conduct an *in vitro* skin irritation test using the diluted chemical and/or the final formulation to ask the question “Is the chemical an irritant at the relevant concentration?”.

If the answer to this question is “yes”, the chemical is an irritant. Depending on comparison with benchmark controls the decision can be made as to acceptability for market or re-formulation of the product. If the outcome is negative the chemical is not an irritant at relevant concentrations. Confirmatory human product testing can be considered to further specify non versus mild/moderate skin irritancy (see below for non-irritant scenario).

If the chemical is an irritant: follow Step 2 above.

If the chemical is non-irritant: Currently, it is not possible to distinguish a mild/moderate irritant from a non-irritant *in vitro* (depicted in Fig. 2 with dotted lines) and assess the whole severity range of irritant responses of non-irritant chemicals. Therefore, confirmatory human product testing can be considered to further specify non versus mild/moderate skin irritancy. This could be patch testing or other appropriate in-use scenario test, considering a stepwise approach to concentrations.

4. Examples of the use of *in vitro* assays as part of the decision tree approach

Numerous citations in the literature state a good correlation between *in vitro* and clinical data. However, there is a regulatory concern that the skin *in vitro* assays are under-predicting positive responses. On the other hand there are reports that they might actually over-predict responses (van de Sandt et al., 1999; Spielmann et al., 2007). One positive aspect of a model that over-predicts a response is that there are likely to be fewer false negatives. This was supported by the ECVAM validation study of the EpiSkin™ model, which predicted only a 1% frequency of false negatives compared to 22.9% of false positives, based on the data set generated in the evaluation (Spielmann et al., 2007). Importantly, validated *in vitro* corrosivity and irritation tests using RHE skin

models provide the same classification information for ingredients as the *in vivo* test. In-house validation studies of the SkinEthic™ RHE model and the SkinEthic™ Oral Mucosal Model (Rowland et al., 2003, 2007) have shown promising results. These models are used routinely in-house to deliver clinical proof of principle at the earliest opportunity. Data generated from these models may be more clinically relevant than data derived from an animal study or human skin patch test, since preclinical or clinical results from these models are not always considered representative of the intended application or use of certain products, particularly in the instance of oral care applications. As a result of the confidence in the alternative models to predict skin irritation, they are already used to assess the safety of chemicals in certain cases. Other reasons for not using animal testing for safety assessment include:

- When the assessment is on finished products, which is banned (EU, 2003).
- When the chemical is an old ingredient for which data are already available. In these cases, a WoE approach can be adopted.
- For low concentrations of chemicals where an exposure-based assessment can be done e.g., a TTC-like approach. This approach is typically used for in-house assessments but the sharing of data between companies should be encouraged.
- Where a WoE approach can be used based on:
 - data from suppliers/literature and historical *in vivo* data;
 - read-across from structurally similar chemicals with known skin irritation potential or historical *in vivo* data (see Section 2.3.3);
 - physicochemical properties indicating low potential for skin irritancy;
 - a documented history of safe use in relevant applications and/or experience or use in other industries.
- Where *in vitro* data compare with product formulations previously assessed as safe, plus a human test for confirmation can be used.
- Where other animal data are available (e.g., skin sensitization) that could help in the safety assessment.

5. Future needs

5.1. Regulatory acceptance of the EpiSkin™, EpiDerm™ and SkinEthic™ models

The SCCP raised concerns about the applicability domain of the validated *in vitro* EpiSkin™ model (SCCP, 2007), and these concerns are currently being addressed. Acceptance by the SCCP of EpiSkin™ data to predict the irritation potential of cosmetic ingredients can therefore be anticipated and to date there have been no comments on the other two models. In consideration of this point, the difference between qualification and validation requires clarification. Validation is needed for ECVAM approval as obtained for the EpiSkin™ model but qualification may only be needed for in-house verification that an *in vitro* assay is predicting clinical outcomes for the applied risk assessment purpose.

5.2. TTC-like approach

TTC-like approach refers to universal exposure thresholds of chemical exposure below which there is a low probability of an appreciable risk of skin irritation to humans (Kroes et al., 2004, 2007). It is a form of risk evaluation in which uncertainties based from data on other compounds are balanced against the low level of exposure (Munro et al., 2008). The use of this approach should ideally be promoted and validated, especially for chemicals which

are used at low concentrations. More concentrations for dose response correlations are needed to interpret irritation potency. This is because there may well be a threshold for this response, a fact that is crucial for safety assessment.

5.3. Cumulative irritation assays

Current alternative models address only acute skin irritation potential and not cumulative irritation potential. Cumulative irritation is more important for many of the cosmetic-type ingredients since the products tend to be used on a routine basis. However, the development of *in vitro* models for chronic irritation is challenging as it is not possible to culture/incubate cells for extended periods (days or weeks). Researchers have been trying to design and validate *in vitro* irritation assays to represent longer exposure periods which are representative of longer (chronic) exposure or to reflect exaggerated use (Rowland et al., 2003). The SkinEthic™ RHE model has been used to compare irritation endpoints (modulation of cell viability, the release and gene expression of IL-1 α and IL-8, and morphological changes) after a 72 h exposure period with irritation observed after 3 weeks in the clinical setting (de Brugerolle de Fraissinette et al., 1999). There was a good correlation between the *in vitro* and *in vivo* irritation potential, however, this model has not been validated. Similarly the validation of other biomarkers of irritation may need to be investigated, as reviewed by Welss et al. (2004).

5.4. Exposure conditions

Extend the use of the *in vitro* methods to reflect the *in vivo* exposure conditions (e.g., rinse-off conditions). Some current dosing protocols include a rinse-off so that the reversibility of the irritation effect can be determined, for example, the validated EpiSkin™ protocol. The question arises as to whether the ‘reversibility’ is currently assessed by *in vitro* models and whether they could be adapted to do this. There may be differences of opinion as to whether this could be achieved, although an ECVAM report by van de Sandt et al. (1999) considered that one of the limitations of *in vitro* models was an inability to assess recovery.

6. Conclusions

Alternative models to the rabbit Draize test to detect skin irritants have been developed and used in the cosmetics industry for a number of years. The potential of a chemical to produce skin corrosion can be evaluated using the validated rat skin TER assay and the Human Skin Model Test (OECD TG 431, 2004) using EpiSkin™, EpiDerm™ and SkinEthic™ RHE models. For skin irritation, EpiSkin™, EpiDerm™ and SkinEthic™ are validated as stand-alone test replacements for the rabbit Draize test. One of the conclusions from the COLIPA workshop of the COLIPA Project Team Safety Assessment 2009/2013, was that the good correlation between *in vitro* and *in vivo* skin irritation, together with the substantial in-house experience of these assays has given confidence in the outcomes of these assays such that in-house safety assessments on new products can be made without the use of animal testing. Decision tree approaches incorporating alternative models for skin corrosion and irritation can be used as a flexible guide for safety assessment, rather than a ‘tick-box’ approach. A decision tree for hazard assessment and labelling, using a WoE approach throughout, involves a step-wise evaluation of firstly, physicochemical characteristics, (Q)SAR and existing data, to identify and rule out corrosive chemicals for further testing; secondly, an *in vitro* corrosivity test; and finally, an *in vitro* irritation test to distinguish between irritants and non-irritants. Once a chemical has been

classified as corrosive, irritant or a non-irritant, its safety assessment can also be evaluated using a second decision tree approach. Corrosive chemicals should be tested in an *in vitro* corrosivity test at the use concentration and, if shown to be non-corrosive, tested for irritation using an RHE *in vitro* irritation model. Chemicals labelled as irritants can be retested at the usage concentration, since they may not be irritants at lower concentrations or when used in the final formulation. Human confirmatory testing of the formulation is only carried out on a case-by-case basis. Future goals include providing further validation data requested by the SCCP to support their acceptance of EpiSkin™; proposing additional work on the TTC-like approach; extending *in vitro* models to include exposure levels (rinse-off); and development of *in vitro* models to reflect chronic exposure. In conclusion, the evaluation of the skin irritation potential of new chemicals for use in cosmetics can be confidently accomplished using only alternative methods.

References

- Balls, M., Amcoff, P., Bremer, S., Casati, S., Coecke, S., Clothier, R., Combes, R., Corvi, R., Curren, R., Eskes, C., Fentem, J., Gribaldo, L., Halder, M., Hartung, T., Hoffmann, S., Schectman, L., Scott, L., Spielmann, H., Stokes, W., Tice, R., Wagner, D., Zuang, V., AM, ECV, 2006. The principles of weight of evidence validation of test methods and testing strategies. The report and recommendations of ECVAM workshop 58. ATLA 34 (6), 603–620.
- Basketter, D.A., Reynolds, F.S., York, M., 1999. Predictive testing in contact dermatitis: irritant dermatitis. Clin. Dermatol. 15, 637–644.
- Benavides, T., Mitjans, M., Martínez, V., Clapés, P., Infante, M.R., Clothier, R.H., Vinardell, M.P., 2004. Assessment of primary eye and skin irritants by *in vitro* cytotoxicity and phototoxicity models: an *in vitro* approach of new arginine-based surfactant-induced irritation. Toxicology 197 (3), 229–237.
- Coquette, A., Berna, N., Poumay, Y., Pittelkow, M.R., 2000. The keratinocyte in cutaneous irritation and sensitization. In: Kydonicis, A.F., Wille, J.J. (Eds.), Biochemical Modulation of Skin Reaction. CRC Press, New York, pp. 125–143.
- Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1 α (IL-1 α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. Toxicol. In Vitro 17 (3), 311–321.
- Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2005. Analysis of interleukin-1 α (IL-1 α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. Toxicol. In Vitro 17 (3), 311–321.
- de Brugerolle de Fraissinette, A., Picarles, V., Chibout, S., Kolopp, M., Medina, J., Burtin, P., Ebelin, M.E., Osborne, S., Mayer, F.K., Spake, A., Rosdy, M., De Wever, B., Ettlin, R.A., Cordier, A., 1999. Predictivity of an *in vitro* model for acute and chronic skin irritation (SkinEthic) applied to the testing of topical vehicles. Cell Biol. Toxicol. 15, 121–135.
- DeLeo, V.A., Carver, M.P., Hong, J., Fung, K., Kong, B., DeSalva, S., 1996. Arachidonic acid release: an *in vitro* alternative for dermal irritancy testing. Food Chem. Toxicol. 34, 167–176.
- ESAC, 2000. Statement on the application of the Corrositex® assay for skin corrosivity testing. 6th December, 2000. Available from: <http://ecvam.jrc.it/publication/CRTX_statement.pdf>.
- ESAC, 2008. Statement on the Scientific Validity of *in vitro* tests for Skin Irritation Testing. Available from: <<http://ecvam.jrc.it/index.htm>>.
- EU, 1967. Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Official J. Eur. Union L196, 1–98.
- EU, 1976. Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products. Official J. Eur. Communities L262, 169–172.
- EU, 2003. EC-Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official J. L66, 26.
- EU, 2008a. 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. Official J. Eur. Union L353, 1–1354.
- EU, 2008b. Council Regulation (EC) No. 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official J. Eur. Union L142, 1–739.
- Faller, C., Bracher, M., Dami, N., Roguet, R., 2002. Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics. Toxicol. In Vitro 16 (5), 557–572.
- Fentem, J.H., Archer, G.E.B., Balls, M., Botham, P.A., Curren, R.D., Earl, L.K., Esdaile, D.J., Holzhtutter, H-G., Liebsch, M., 1998. The ECVAM international validation

- study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicol. In vitro* 12, 483–524.
- Fentem, J.H., Briggs, D., Chesné, C., Elliott, G.R., Harbell, J.W., Heylings, J.R., Portes, P., Roguet, R., van de Sandt, J.J., Botham, P.A., 2001. A prevalidation study on *in vitro* tests for acute skin irritation, results and evaluation by the Management Team. *Toxicol. In vitro* 15 (1), 57–93.
- Gerner, I., Spielmann, H., Hoefler, T., Liebsch, M., Herzler, M., 2004a. Regulatory use of (Q)SARs in toxicological hazard assessment strategies. *SAR QSAR Environ. Res.* 15, 359–366.
- Gerner, I., Barratt, M.D., Zinke, S., Schlegel, K., Schlede, E., 2004b. Use of physicochemical property limits to develop rules for identifying chemical substances with no skin irritation or corrosion potential. *QSAR Combinatorial Sci.* 23, 726–733.
- Grindon, C., Combes, R., Cronin, M.T., Roberts, D.W., Garrod, J.F., 2007. Integrated decision-tree testing strategies for skin corrosion and irritation with respect to the requirements of the EU REACH legislation. *ALTA* 35 (6), 673–682.
- Gueniche, A., Ponc, M., 1993. Use of human skin cell cultures for the estimation of potential skin irritants. *Toxicol. In vitro* 7, 15–24.
- Heylings, J.R., Clowes, H.M., Hughes, L., 2001. Comparison of tissue sources for the skin integrity function test (SIFT). *Toxicol. In vitro* 15 (4–5), 597–600.
- Heylings, J.R., Diot, S., Esdaile, D.J., Fasano, W.J., Manning, L.A., Owen, H.M., 2003. A prevalidation study on the *in vitro* skin irritation function test (SIFT) for prediction of acute skin irritation *in vivo*: results and evaluation of ECVAM Phase III. *Toxicol. In vitro* 17 (2), 123–138.
- Hoffman, J., Heiser, E., Karpinski, S., Losse, J., Thomas, D., Seifken, W., Ahr, H.-J., Vohr, H.-W., Fuchs, H.W., 2005. Epidermal-skin-test 1000 (EST 1000)—a new reconstructed epidermis for *in vitro* skin corrosivity testing. *Toxicol. In vitro* 19, 925–929.
- Jacobs, G.A., Castellazzi, A., Dierickx, P.J., 1989. Evaluation of a non-invasive human and an *in vitro* cytotoxicity method as alternatives to the skin irritation test on rabbits. *Contact Dermatitis* 21 (4), 239–244.
- Johnson, M.A., Maggiora, G.M., 1990. Concepts and Applications of Molecular Similarity. John Wiley & Sons, New York, pp. 393.
- Kandárová, H., Liebsch, M., Spielmann, H., Genschow, E., Schmidt, E., Guest, R., Whittingham, A., Warren, N., Gamer, A.O., Remmele, M., Kaufmann, T., Wittmer, E., De Wever, B., 2004. Assessment of the human epidermis model Skin Ethic RHE™ for *in vitro* skin corrosion testing according to OECD TG 431. *ALTEX* 21, 160–161.
- Kandárová, H., Liebsch, M., Schmidt, E., Genschow, E., Traue, D., Spielmann, H., Meyer, K., Steinhoff, C., Tornier, C., De Wever, B., Rosdy, M., 2006. Assessment of the skin irritation potential of chemicals by using the SCCP, 2006. The SCCP's notes of guidance for the testing of cosmetic ingredients, their safety evaluation 6th revision. Adopted by the SCCP during the 10th plenary meeting of 19 December 2006. SkinEthic reconstructed human epidermal model and the common skin irritation protocol evaluated in the ECVAM skin irritation validation study. *ALTA* 34 (4), 393–406.
- Kaufmann, T., Remmele, M., Holzthutter, H.G., 2000. The ECVAM prevalidation study on the use of EpiDerm™ for skin corrosivity testing. *ALTA* 28, 371–401.
- Kroes, R., Renwick, A.G., Cheeseman, M., Kleiner, J., Mangelsdorf, I., Piersma, A., Schiller, B., Schlatter, J., van Schothorst, F., Vos, J.G., Wurtzen, G., 2004. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. *Food Chem. Toxicol.* 42 (1), 65–83.
- Kroes, R., Renwick, A.G., Feron, V., Galli, C.L., Gibney, M., Greim, H., Guy, R.H., Lhuguenot, J.C., van de Sandt, J.J., 2007. Application of the threshold of toxicological concern (TTC) to the safety evaluation of cosmetic ingredients. *Food Chem. Toxicol.* 45 (12), 2533–2562.
- Lee, J.K., Kim, D.B., Kim, J.L., Kim, P.Y., 2000. *In vitro* cytotoxicity tests on cultured human skin fibroblasts to predict skin irritation potential of surfactants. *Toxicol. In vitro* 14 (4), 345–349.
- Mombelli, E., 2008. An evaluation of the predictive ability of the (Q)SAR software packages, DEREK, HAZARDEXPERT and TOPKAT, to describe chemically-induced skin irritation. *ALTA* 36 (1), 15–24.
- Müller-Decker, K., Furstemberger, G., Marks, F., 1994. Keratinocyte-derived pro-inflammatory key mediators and cell viability as *in vitro* parameters of irritancy: a possible alternative to the Draize skin irritation test. *Toxicol. Appl. Pharmacol.* 127, 99–108.
- Munro, I.C., Renwick, A.G., Danielewska-Nikiel, B., 2008. The threshold of toxicological concern (TTC) in risk assessment. *Toxicol. Lett.* 180 (2), 151–156.
- OECD TG 404, 2002. Guideline for Testing of Chemicals, No. 404: Acute Dermal Irritation, Corrosion. Revised Test Guideline as adopted 24 April, plus Annexes, 2002, 7 pp.
- OECD TG 430, 2004. Guideline for Testing of Chemicals, No. 430: *In vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER). Original Guideline. (accessed 13.04.04.).
- OECD TG 431, 2004. Guidelines for the Testing of Chemicals, No. 431: *In vitro* Skin Corrosion: Human SkinModel Test. Original Guideline, Paris, France, OECD, 8 p. (accessed 13.04.04.).
- OECD TG 435, 2004. Guidelines for the Testing of Chemicals, No. 435 DRAFT: *In Vitro* Membrane Barrier Test Method for Skin Corrosion (1st Version).
- Oliver, G.J.A., Pemberton, M.A., Rhodes, C., 1986. An *in vitro* skin corrosivity test/modifications and validation. *Food Chem. Toxicol.* 24, 507–512.
- Perkins, M.A., Osborne, R., Rana, F.R., Ghassemi, A., Robinson, M.K., 1999. Comparison of *in vitro* and *in vivo* human skin response to consumer products and ingredients with a range of irritancy potential. *Toxicol. Sci.* 48, 218–229.
- Rispin, A., Harbell, J.W., Klausner, M., Jordan, F.T., Coecke, S., Gupta, K., Stitzel, K., 2004. Quality assurance for *in vitro* alternative test methods: quality control issues in test kit production. *ALTA* 32 (S1B), 725–729.
- Rorije E., Hulzebos E., 2005. Evaluation of (Q)SARs for the prediction of skin irritation/corrosion potential physico-chemical exclusion rules. National Institute of Public Health and Environment (RIVM) report, 2005.
- Rowland, J.L., Cappadoro, M., De Wever, B., Davis, A., Freeman, S., 2003. Results of a skin irritation validation study with topical acne formulations. *Toxicol. Lett.* 144, 156.
- Rowland, J.L., Cappadoro, M.A., Rees, G.D., 2007. Development & Validation of an *In vitro* Oral Soft Tissue Model for Assessing Potential Toxicity of Actives & Excipients. Presented at the 4th International Workshop September 6 and 7, 2007, Nice, France.
- Saliner, A.G., Tsakovska, I., Pavan, M., Patlewicz, G., Worth, A.P., 2007. Evaluation of SARs for the prediction of skin irritation/corrosion potential: structural inclusion rules in the BfR decision support system. *SAR QSAR Environ. Res.* 18 (3–4), 331–342.
- Sanchez, L., Mitjans, M., Infante, M.R., Vinardell, M.P., 2006. Determination of interleukin-1 α in human NCTC 2544 keratinocyte cells as a predictor of skin irritation from lysine-based surfactants. *Toxicol. Lett.* 167 (1), 40–46.
- SCCP, 2007. Memorandum on the *in vitro* test EpiSkin™ for skin irritation testing, SCCP/1145/07, Scientific Committee on Consumer Products.
- Schäfer-Korting, M., Bock, U., Diembeck, W., Düsing, H.J., Gamer, A., Haltner-Ukomadu, E., Hoffmann, C., Kaca, M., Kamp, H., Kersen, S., Kietzmann, M., Korting, H.C., Krächter, H.U., Lehr, C.M., Liebsch, M., Mehling, A., Müller-Goymann, C., Netzlaff, F., Niedorf, F., Rübbecke, M.K., Schäfer, U., Schmidt, E., Schreiber, S., Spielmann, H., Vuia, A., Weimer, M., 2008. The use of reconstructed human epidermis for skin absorption testing: results of the validation study. *ALTA* 36 (2), 161–187.
- Smith, J.S., Macina, O.T., Sussman, N.B., Luster, M.I., Karol, M.H., 2000. A robust structure-activity relationship (SAR) model for esters that cause skin irritation in humans. *Toxicol. Sci.* 55 (1), 215–222.
- Spekstra, S.W., Toebak, M.J., Sampat-Sardjoeperad, S., van Beek, P.J., Boorsma, D.M., Stooft, T.J., von Blomberg, B.M.E., Scheper, R.J., Bruynzeel, D.P., Rustemeyer, T., Gibbs, S., 2005. Induction of cytokine (interleukin-1 α and tumor necrosis factor- α) and chemokine (CCL20, CCL27, and CXCL8) alarm signals after allergen and irritant exposure. *Exp. Dermatol.* 14, 109–116.
- Spielmann, H., Hoffmann, S., Liebsch, M., Botham, P., Fentem, J.H., Eskes, C., Roguet, R., Cotovio, J., Cole, T., Worth, A., Heylings, J., Jones, P., Robles, C., Kandárová, H., Gamer, A., Remmele, M., Curren, R., Raabe, H., Cockshott, A., Gerner, I., 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. *ALTA* 35 (6), 559–601.
- Stobbe, J.L., Drake, K.D., Maier, K.J., 2003. Comparison of *in vivo* (Draize method) and *in vitro* (Corrositex assay) dermal corrosion values for selected industrial chemicals. *Int. J. Toxicol.* 22 (2), 99–107.
- Tornier, C., Rosdy, M., Maibach, H.I., 2006. *In vitro* skin irritation testing on reconstituted human epidermis: reproducibility for 50 chemicals tested with two protocols. *Toxicol. In vitro* 20 (4), 401–416.
- van de Sandt, J.R., Roguet, C., Cohen, D., Esdaile, M., Ponc, E., Corsini, C., Barker, N., Fusenig, M., Liebsch, Benford, D., Bruggerolle de Fraissinette, A., Fartasch, M., 1999. The use of human keratinocytes and human skin models for predicting skin irritation. *ATLA* 27, 723–743.
- Wells, T., Basketter, D.A., Schröder, K.R., 2004. *In vitro* skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicol. In vitro* 18 (3), 231–243.
- Williams, I.R., Kupper, T.S., 1996. Immunity at the surface. homeostatic mechanisms of the skin immune system. *Life Sci.* 58 (18), 1485–1507.
- York, M., Griffiths, H.A., Whittle, E., Basketter, D.A., 1996. Evaluation of a human patch test for the identification and classification of skin irritation potential. *Contact Dermatitis* 34, 204–212.