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

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ABSTRACT

The need for alternative approaches to replace the *in vivo* rabbit Draize eye test for evaluation of eye irritation of cosmetic ingredients has been recognised by the cosmetics industry for many years. Extensive research has lead to the development of several assays, some of which have undergone formal validation. Even though, to date, no single *in vitro* assay has been validated as a full replacement for the rabbit Draize eye test, organotypic assays are accepted for specific and limited regulatory purposes. Although not formally validated, several other *in vitro* models have been used for over a decade by the cosmetics industry as valuable tools in a weight of evidence approach for the safety assessment of ingredients and finished products. In light of the deadlines established in the EU 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. Furthermore, recommendations are given on how remaining data gaps and research needs can be addressed.

1. Introduction

According to the Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals Test No. 405: acute eye irritation/corrosion, eye irritation is defined as "... the production of changes in the eye following application of a test substance to the anterior surface of the eye, which are fully reversible within 21 days of application". The same guideline defines eye corrosion as "... the production of tissue damage in the eye, or serious physical decay of vision, following application of a test substance to the anterior surface of the eye, which is not fully reversible within 21 days of application" (OECD TG 405, 2002). Different regulatory systems exist, e.g., within the European Union (EU) (EU, 2004), United States and on a more global basis (UN, 2003) which classify substances based on the severity and persistence of the eye responses (cornea, iris and conjunctiva) that they produce. Such classifications translate into labelling of the substance and for products where required by legislation.

In general, topical eye irritants cause local effects on the front structures of the eye e.g., cornea, conjunctiva, iris and lachrymal system. The extent of involvement of these different ocular structures in irritation is a reflection of the severity of the response. Typically, slight irritants produce primarily conjunctival effects with little or no corneal involvement. While conjunctival responses generally precede corneal responses, corneal injury is associated with

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moderate and severe irritation responses. Research into the *in vivo* mechanistic basis for ocular irritation using different chemical classes comprising surfactants (anionic, cationic, and non-ionic), acids, alcohols, aldehydes, alkalis and bleaches have shown that depth of injury to the cornea, in the early hours after exposure, is predictive of the eventual degree and duration of the ocular lesions in the rabbit (Maurer et al., 2002; Jester, 2006). This research demonstrated that slight irritants tend to affect only the superficial corneal epithelium, mild and moderate irritants affect epithelium and superficial stroma whilst highly moderate and severe irritants affect deeper layers of the stroma (and possibly the endothelium). In turn, the depth of injury is also related to the eventual degree and recovery of the injury. Common mechanisms of injury causing acute effects include membrane lysis, protein coagulation, saponification and action on macromolecules. Chemicals that react with nucleic acids, mitochondrial proteins, or other cellular targets often show a longer latency period between exposure and maximum manifestation of damage to the cornea (Maurer et al., 2002; Jester, 2006).

Cosmetics may come into contact with the eye under conditions of intended use or accidental exposure (e.g., in the case of mascaras and shampoos, respectively). Both scenarios need to be evaluated in a proper safety assessment, as stipulated in the EU Cosmetics Directive (EU, 1976). Due to this potential exposure, it is essential to assess the ocular safety of cosmetic ingredients and/or final cosmetic products. The rabbit Draize eye test (OECD TG 405, 2002) is globally accepted as the standard regulatory method for evaluating the eye irritation potential of substances and has been used for several decades. An extensive number of *in vitro* models have been developed and proposed as alternatives to the rabbit Draize eye test. A overview of these methods is available in a comprehensive review published by Eskes et al. (2005). Several of these *in vitro* assays have been included in six major validation or evaluation studies (EC/HO (Balls et al., 1995), COLIPA (Brantom et al., 1997), BGA/BMBF (Spielmann et al., 1993, 1996), CTFA (Gettings et al., 1991, 1994, 1996), IRAG (Bradlaw et al., 1997) and MHW/JCIA (Ohno et al., 1994)) that took place between 1991 and 1997. A review of these studies (Balls et al., 1999) concluded that despite good reproducibility and sensitivity of several of the *in vitro* assays for ocular irritation, the predictive performance of each individual assay was not sufficient to fully replace the rabbit Draize eye test. Despite this, organotypic assays (models that resemble the *in vivo* situation in 3-D form or function or both) are widely used for specific, limited regulatory purposes. The Bovine Corneal Opacity and Permeability (BCOP),[1] Isolated Chicken Eye (ICE), Isolated Rabbit Eye (IRE) and the Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM) have been officially accepted since 2004 by European authorities for the classification and labelling of severe eye irritants. More recently, the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC) issued statements of scientific validity for BCOP and ICE as screening tests for identification of ocular corrosives and severe eye irritants (ECVAM, 2007). These statements support the outcome of the Interagency Co-ordinating Committee for the Validation of Alternative Methods (ICCVAM) Background Review Document activities for these organotypic assays (ICCVAM, 2006). In order to identify irritants over the entire potency range for all chemical classes, it is generally accepted that a battery of alternative assays will be required. Furthermore, the cosmetics industry has a need for *in vitro* assays that provide greater resolution and precision in the mild to very mild range of eye irritancy than are offered by the standard rabbit Draize eye test.

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 2003), 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 eye irritation after March 2009 was analysed at a COLIPA scientific meeting organised by its Safety Assessment and Eye Irritation Project Teams in Brussels on 30th January, 2008. Participants included safety experts from a number of cosmetic companies. Decision trees for safety assessment were developed using the outcome of the discussions held during the meeting, in which tiered testing strategies and the use of weight-of-evidence (WoE) were considered major principles. Gaps and hurdles were also identified and recommendations for further activities were developed.

2. Results and discussion

2.1. Current alternative approaches to the assessment of eye irritation

Current safety assessment practices make routine use of tiered testing strategies based on a WoE approach. WoE approaches have long been in use 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 eye irritation. Such information may include, for example:

- Physicochemical properties.
- Historical *in vivo* animal data.
- *In vitro* data.
- Human data (clinical and post-market surveillance).
- Exposure.

If the information which is initially available is considered insufficient, a tiered testing strategy is pursued that allows for the generation of additional data. Important elements may include read-across approaches based on chemical domain (OECD Application Toolbox (www.oecd.org)). Integrated testing strategies have been applied in the chemical sector, and were recently re-evaluated in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) context (Grindon et al., 2008), as well as the application of WoE approaches (e.g., OSIRIS project) (van Leeuwen et al., 2007).

[1] BCOP, Bovine Corneal Opacity and Permeability; BfR, Bundesinstitut für Risikobewertung (German Federal Institute for Risk Assessment); BGA, Bundesgesundheitsamt (German Department of Research and Technology); BMBF, Bundesministerium für Bildung und Forschung (German Federal Ministry of Education and Research); COLIPA The European Cosmetic Association; CTFA, Cosmetic, Toiletry and Fragrance Association; DSS, Decision Support System; EC/HO, European Commission/British Home Office; ECVAM, European Centre for the Validation of Alternative Methods; EPAA, European Partnership on Alternative Approaches to Animal Testing; ESAC, ECVAM Scientific Advisory Committee; EU, European Union; GHS, Globally Harmonised System; HCE, Human corneal epithelium; HET-CAM, Hen's Egg Test on the Chorio-Allantoic Membrane; ICCVAM, Interagency Co-ordinating Committee for the Validation of Alternative Methods; ICE, Isolated Chicken Eye; IRAG, Interagency Regulatory Alternatives Group; MHW/JCIA, (Japanese) Ministry of Health and Welfare/Japanese Cosmetic Industry Association; NC, not classified; OECD, Organisation for Economic Co-operation and Development; (Q)SAR, (Quantitative) Structure Activity Relationship; Reconstructed human Tissue (RhT); REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; TTC, Threshold of Toxicological Concern; WoE, weight of evidence.

2.2. Alternative approaches already applicable as elements in a WoE approach

There are several different data sources that are incorporated into a WoE approach to evaluate eye irritation. A brief description of how the information available from each of these data sources is useful in a WoE approach to evaluate eye irritation without the use of newly generated animal data is provided below.

2.2.1. Physicochemical properties

Information of relevance to eye irritation/corrosion can be inferred from basic physicochemical characteristics of a substance such as, but not limited to physical form (liquid, solid, gel), solubility, pH, pKa and molecular weight, ionisation potential, vapour pressure, water solubility, critical micelle concentration. For example, substances with 'extreme' pH values ($\geqslant 11.5$ or $\leqslant 2$) may have strong local effects such as severe eye irritation/corrosion. Several studies have investigated and confirmed the usefulness of pH as a predictor of corrosion (Worth and Cronin, 2001) and as an element in tiered testing strategies (Worth, 2004). However, where extreme pH is the only basis of identification of a corrosive, it may also be important to take into consideration the acid/alkaline reserve, a measure of the buffering capacity of a chemical substance (Botham et al., 1998; Young et al., 1994). The use of physicochemical properties as a starting point in a tiered strategy for evaluation of eye irritation is provided as a supplement in the OECD guideline 405 for the testing of chemicals, acute eye irritation/corrosion (OECD TG 405, 2002).

2.2.2. Historical in vivo animal data

Availability of historical animal data for chemicals with high structural similarity is often useful for the eye irritation assessment of a substance within the same chemical domain. When animal data are available on similar substances these data can be used in a read-across approach to demonstrate that a substance under consideration that is of the same chemical class is likely to produce the same ocular responses. Such an evaluation is 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 ocular effects.

Validated and accepted (Q)SAR approaches for eye irritation are currently not available, in part due to eye irritation being a difficult endpoint to model *in silico* due to the complexity of the biological mechanisms that may be involved in eye irritation.

2.2.3. In vitro data

There are a number of *in vitro* assays used within the industry for purposes of screening and safety assessment of substances for eye irritation. Tables 1 and 2 list a number of *in vitro* eye irritation assays, together with a brief description of the assays, information that is obtained from them, their validation status, and their applicability based on current practices and scientific literature. Table 1 lists assays with regulatory acceptance and Table 2 without regulatory acceptance.

In vitro tests have been used routinely by cosmetic companies for over a decade. They are typically used for early routine screening in the development process of new ingredients/products and provision of safety assessment as part of an integrated testing strategy for ingredients and finished products as they proceed to market. The *in vitro* assays are ideally used, usually in combination, to assess classes of materials where the mode of action (e.g., cell death, coagulation, saponification, interaction with macromolecules) to effect eye irritation is well understood. Confidence for such in-house use of these *in vitro* assays is dependent on a number of factors such as the availability of appropriate benchmarks, historical information on similar materials, an understanding of the limitations of the assay(s) and the technical expertise of the user. For these reasons, such use of *in vitro* methods and their combination is often company-specific. As such, application of these methods has allowed companies to eliminate finished formulation testing using animals over a decade ago and to evaluate to a large extent the eye irritation of ingredients in cosmetic products.

2.2.4. Human data

2.2.4.1. Clinical studies. Human volunteer studies can be an integral part of the overall safety assessment of a cosmetic product. This is because it is possible to evaluate product compatibility in humans by using clinical evaluations under the anticipated and foreseeable uses.

Such studies are always confirmatory in nature and are not hazard identification studies. They can only be conducted when the WoE has demonstrated that a product is safe for eye exposure but the judgement is that additional confirmatory data would be useful to the overall product safety assessment.

Ethical considerations, involvement of qualified personnel (e.g., ophthalmologists) and consideration of Good Clinical Practice (GCP) guidelines and availability of *in vitro* eye irritation tests as part of the pre-clinical evaluation are of paramount importance when considering the conduct of human volunteer studies. Such human volunteer studies may involve direct eye instillation or repeated peri-ocular application by the study investigator or product use by the study participant under normal use conditions. In all cases, objective assessment by an ophthalmologist and subjective assessment by the study participant is conducted.

2.2.4.2. Human experience. Human experience can be derived from different sources such as from in-house post-market surveillance systems in which individual companies monitor the marketplace for the occurrence of adverse event through consumer contact typically through provision of an on-pack contact number for the company. In addition, human experience can be derived from industry-wide consumer follow-up systems and from national Poison Control Centres (PCCs) on reported accidental exposures.

2.2.5. Exposure

Finished products are a combination of several different chemicals each present at a specific concentration. As such, it is important to understand the eye irritation potential of the ingredients at in-use concentrations in addition to the neat form. Exposure considerations that need to be taken into account are type of formulation (e.g., skin care, shampoo), area of application (e.g., face, scalp, axilla, body) and frequency of application (several times a day, intermittently). In cases where very low concentrations of chemicals are present in a finished product, an exposure-based assessment may allow the conclusion that any local effects such as eye irritation are highly unlikely to occur.

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

2.3.1. COLIPA eye irritation programme

To address development of alternative methods based on mechanistically relevant biological events, the European Cosmetics Association (COLIPA) through its overall Alternatives to Animal Testing (AAT) initiative has in place a programme for the development of *in vitro* assays for eye irritation that is managed by the Project Team PT-Eye Irritation. The overall programme incorporates three core elements: (1) method development/optimisation of existing models to validation; (2) collaborative activities with external partners; and (3) integrated research projects that are conducted in collaboration with academia. The short-term need

Table 1
A summary of *in vitro* assays with official regulatory acceptance.

	Assay			
	Bovine Corneal Opacity and Permeability (BCOP) assay	Isolated Chicken Eye (ICE) formerly known as Chicken Enucleated Eye Test (CEET)	Isolated Rabbit Eye (IRE) assay	Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM) assay (different protocols)
Ocular effect	Corneal damage	Corneal damage	Corneal damage	Conjunctival damage
Evaluation end point	Corneal opacity, thickness and permeability. Histopathology recommended to be incorporated as a routine endpoint	Corneal opacity, swelling and permeability Histopathology recommended to be incorporated as a routine endpoint	Corneal opacity, swelling and integrity, barrier function. Histopathology recommended to be incorporated as a routine endpoint	Vascular changes in the egg's CAM (haemorrhage, lysis) and coagulation, (hyperaemia)
Validation status	ECVAM Statement of scientific validity for identification of severe irritants/ocular corrosives (April 2008). Additional data review by ECVAM/ ICCVAM to evaluate usefulness of BCOP to identify levels of irritancy lower than severe	ECVAM Statement of scientific validity for identification of severe irritants/ocular corrosives (April 2008). Additional data review by ECVAM/ ICCVAM to evaluate usefulness of ICE to identify levels of irritancy lower than severe	None (further review is recommended)	None (further review is recommended; a retrospective validation of HET-CAM data is currently under discussion)
Regulatory acceptance	*Severe eye irritants/ocular corrosives (GHS Cat 1/R41 classification)* Accepted by EU to identify ocular corrosives and severe irritants (July 2004) Accepted by US regulatory agencies to identify ocular corrosives and severe irritants (June 2008) On-going development of an OECD guideline based on the current regulatory acceptance (for severe irritant/ocular corrosive)	*Severe eye irritants/ocular corrosives (GHS Cat 1/R41 classification)* Accepted by EU to identify ocular corrosives and severe irritants (July 2004) Accepted by US regulatory agencies to identify ocular corrosives and severe irritants (June 2008) On-going development of an OECD guideline based on the current regulatory acceptance (for severe irritant/ocular corrosive)	*Severe eye irritants/ocular corrosives (GHS Cat 1/R41 classification)* Accepted by EU to identify ocular corrosives and severe irritants (July 2004)	*Severe eye irritants/ocular corrosives (GHS Cat 1/R41 classification)* Accepted by EU to identify ocular corrosives and severe irritants (July 2004)
Irritancy range detected according to literature	Moderate, severe and very severe. Histology proposed to improve predictive capacity for severe irritants identified as false negatives[1] and for greater discrimination of mild/moderate irritants such as surfactants and surfactant-based products[2] surfactant-based rinse-off personal care [2]	Severe and non-irritants[1,3]	Severe[1] Mild to moderate for surfactant-based formulations [1,4,5]	Mild to non-irritating, different protocols have been adapted to materials with different physico-chemical properties[1] Severe irritants [6,7]
Chemical class according to literature	Wide range of physical forms and solubilities[1] Chemicals (alcohols, ketones, carboxylic acids, heterocyclic compounds over-predicted) and formulations[1]. Chemicals and formulations associated with highly fragranced products[1] Oxidisers (hair dye formulations)[1]. Personal care products (e.g., shampoos, deodorants)[1] Surfactant-based formulations[8]	Wide range of physical forms and solubilities such as liquids, pastes and gels. Surfactant-based formulations[3]	Alkaline materials, anionic and cationic surfactants[1]. Formulations in the cosmetic and pharmaceutical industries[1]. Surfactant-based formulations[1,4]	Surfactants[7]. Many types of ingredients and formulations (rinse-off, leave on, water soluble/ non-soluble, solids)[6,7] especially suited for surfactants and surfactant-based formulations[6], biological membranes (dyes, pigments)[1,6,7]
Limitations	Not as sensitive in distinguishing between mild irritants with the standard protocol[1] Underestimation of substances acting primarily on the iris or the conjunctiva[1] unless combined with histology[2]	Possible limitation for solids[1]	Possible limitation for solids[1]	Possible limitation for solids and with substances that stain[1]

To note: Table 1 is a compilation of recent information abstracted from the scientific literature. As such, it is meant to provide the reader with an overview of the type and quantity of information available in the public domain on reported uses of *in vitro* eye irritation assays. Table 1 is not meant to be a comprehensive overview of all of the information available. Information available up to 2005 is comprehensively reviewed in Eskes et al. (2005).
References are cited in Table 1 using a suffixed number. The full citation is provided in the reference section.
[1]Eskes et al. (2005), [2]Cater and Harbell (2006), [3]Prinsen (1996), [4]Cooper et al. (2001), [5]Jones et al. (2001), [6]Spielmann et al. (1997), [7]Steiling et al. (1999), [8]Cater and Harbell (2008), [9]Bagley et al. (1994).

Table 2
A summary of *in vitro* assays without official regulatory acceptance so far.

Assay	Ocular effect	Evaluation end point	Validation status	Reported uses in the peer-reviewed scientific literature	
				Irritancy range detected	Chemical class
Chrioallantoic Membrane Vascular assay (CAMVA)	Conjunctival damage	Vascular changes in the isolated CAM: Haemorrhaging, hyperaemia, inhibition of blood flow	None	Best for mild to moderate irritation; limitations at the lower end and for severe irritants[1,4]	Broad range of formulations (rinse-off and leave-on) and ingredients (solvents, alcohols, surfactants, acids, bases)[2,3,4]
Human Corneal Epithelium (HCE) SkinEthic™	Corneal damage	Cytotoxicity based on MTT reduction expressed either as% viability or as ET_{50}	Entered into a formal validation study (end 2008). ESAC peer review expected in 2009	Slightly irritant, moderately irritant and irritant[5,6] Irritant versus non-irritant[6] Non-irritating, very slightly irritating, slightly irritating, irritating and very irritating[7]	Broad range of chemicals and product formulations of different physical form including liquids and solida[5,7,8,9,10]. Raw materials (surfactants esters, alcohols, ketones, miscellaneous) and cosmetic products (alcohol-based products, bath and cleaning products, skin and suncare products make-up products[5] Hydrophilic and hydrophobic chemicals, dyes, polymers, silicones, solvents[6] Surfactants[5,7,8,9,10]. Surfactant-based shampoos and conditioner[6]. Raw materials including surfactants, polymers silicones, dyes, solvents, vegetable extracts, preservatives[9,10]
MatTek EpiOcular™ assay	Corneal damage	Cytotoxicity based on MTT reduction expressed either as% cell viability or as ET_{50}	Entered into a formal validation study (end 2008) ESAC peer review expected in 2009	Mild to moderate[1] Non-irritant versus irritant[11] Mild to moderate and potentially severe	Broad range of chemicals and product formulations of different physical form including hydrophilic and hydrophobic materials[1,8,11,12]. Oversensitive to alcohols and esters. Not appropriate for highly volatile liquids, organic solvents and certain classes of reactive chemicals (e.g., peroxides)[1]. Raw materials (surfactants, hydrocarbons, amines, esters and ketones)[11]. Finished products (shampoo, bar soap, hair conditioner, skin creams, toothpaste antiperspirant/deodorant)[12] Surfactant-based shampoos[13,14] and shampoos and conditioners[8]
Neutral Red Release assay (different cell types possible)	Corneal damage	Cytotoxicity measured by release of the dye Neutral Red into a mono-layer cell cultures (plasma membrane damage, loss of lysosomal integrity)	Ongoing Validation Management Group (VMG) review for post hoc validation ESAC peer review expected in 2009	Especially suited for the mild to very mild range[1] Accepted by local authorities in France for evaluation of eye irritation of finished products	Especially suited for surfactants and surfactant-based and water-soluble or miscible substances and formulations, including personal care materials[1,15], Wide range of cosmetic formulations (leave-on and rinse-off) and ingredients (alcohols, surfactants, ketone, bases, acids, esters)[16,17]. Hair-care formulations: shampoos and conditioners[8]
Neutral Red Uptake assay	Corneal damage	Cytotoxicity measured by inhibition of uptake of the dye Neutral Red into mono-layer cell cultures	Not subject to validation	Mild irritants[1]	Surfactants[1,18,19]. Limitations for substances with extremely high or low pH, high reserve acidity or alkalinity, highly volatile, coloured[1]
Red Blood Cell (RBC) assay	Corneal damage	Cytotoxicity based on haemolysis and oxyhaemaglobin denaturation in Red Blood Cells	Ongoing Validation Management Group (VMG) review for post hoc validation ESAC peer review expected in 2009	Very mild and non-mild[1]	Surfactants and surfactant-based formulations such as shampoos and personal care products[7,19,20,21,22,23,24]
Fluorescein Leakage assay or Trans-Epithelial Permeability (TEP) assay	Corneal damage	Permeability of fluorescein through a monolayer of epithelial cells in culture that have formed tight junctions and desmosomes	Ongoing Validation Management Group (VMG) review for post hoc validation ESAC peer review expected in 2009	Mild to moderate[1]	Surfactants, surfactant-based formulations[1,18,25,26] and alcohols[8,19]

(*continued on next page*)

Table 2. (*continued*)

Assay	Ocular effect	Evaluation end point	Validation status	Reported uses in the peer-reviewed scientific literature	
				Irritancy range detected	Chemical class
Cytosensor formerly known as Silicon Microphysiomer (SM) assay	Ocular irritation	Changes in surface potential changes measured as pH resulting from inhibition of metabolic rate (release of acidic metabolites from cultures cells)	Ongoing Validation Management Group (VMG) review for post hoc validation ESAC peer review expected in 2009	Innocuous-mild, mild-moderate and moderate-severe[1]	Surfactants and surfactant-based formulations e.g., personal care products[1,19,27,28]
Slug Mucosal Irritation (SMI) assay	Ocular irritation	Release of mucus, lactate dehydrogenase, proteins and alkaline phosphatise from the mucosal surface of the slug	Submitted to ECVAM by method developer for consideration of formal validation	Non-irritant, irritant and severe[1]	Raw materials (surfactants esters, alcohols, ketones, miscellaneous[29,30]
Short Time Exposure (STE) Test	Corneal damage	Cell viability of SIRC cells	None	Minimally irritant, moderate irritant to severe irritant	Raw materials; surfactants, alcohols, amines, acids, ketones, organic salts, polyols, alkane, inorganic, inorganic base, ester, hydrocarbon, PABA derivative, colour additive[31]

To note: Table 2 is a compilation of recent information abstracted from the scientific literature (see Table 1 legend).
References are cited in Table 1 using a suffixed number. The full citation is provided in the reference section.
[1]Eskes et al. (2005), [2]Bagley et al. (1994), [3]Spielmann et al. (1997), [4]Bagley et al. (1999), [5]Doucet et al. (2006), [6]Van Goethem et al. (2006), [7]Mehling et al. (2007), [8]Jones et al. (2001), [9]Cotovio et al. (2007), [10]Cotovio et al. (2008), [11]Kaluzny et al. (2008), [12]Osborne et al. (1995), [13]Blazka et al. (2003), [14]Blazka et al. (2006), [15]Bradlaw et al. (1997), [16]Courtellemont et al. (1999), [17]Brantom et al. (1997), [18]Jones et al. (1999), [19]Harbell et al. (1997), [20]Pape et al. (1987), [21]Pape et al. (1999), [22]Lagarto et al. (2006), [23]Pape and Hoppe (1990), [24]Pape and Hoppe (1991), [25]Cottin and Zanvit (1997), [26]Zanvit et al. (1999), [27]Bruner et al. (1991), [28]Harbell et al. (1999), [29]Adriaens (2006), [30]Adriaens et al. (2008), [31]Takahashi et al. (2008).

of the cosmetics industry to have validated *in vitro* assays available as soon as possible are addressed by parts 1 and 2 whilst the longer term need to have available *in vitro* assays based on a better understanding of mechanisms of eye irritation is addressed by part 3 of the programme.

Brief details of the approach and projects within the core elements of the COLIPA programme are:

2.3.1.1. Method development/optimisation of existing models. The method development/optimisation of current *in vitro* assays within the COLIPA PT-SCAAT (Project Team of the Steering Committee on Alternatives to Animal Testing) eye irritation programme is focused on Reconstructed human Tissue (RhT) models. Several RhT models are available with some being more advanced in both development and availability than others.

- Two of the most advanced models currently available are the SkinEthic™ Human Corneal Epithelium (HCE) model (derived from Human Corneal Epithelium) and the MatTek Epiocular™ model (derived from human keratinocytes). Recent work on these models has been led by industry in collaboration with the test method developers to expand the data available for predictive capacity and reproducibility (within and between laboratories) using optimised protocols. COLIPA is now engaged with ECVAM in a formal validation study for these two RhT models.
- Other RhT eye irritation models under development have reported improved barrier function capability over some of the commercially available models. COLIPA is engaged in understanding whether use of barrier function as an endpoint is useful to measure in an RhT assay for evaluation of eye irritancy at the lower (non-irritant/very mild) end of the irritancy range.

2.3.1.2. Integrated analysis to define batteries of in vitro assays. All assays have specific domains of applicability whether this relates to range of irritancy or to types of chemical classes. If the determined applicability domain does not fully cover the purpose of the safety assessment, combinations of assays are likely to be needed. Today, combinations of *in vitro* assays are used by individual companies as an integral part of their safety assessments (see Section 4). Likewise, validation efforts focus increasingly on testing batteries since it is generally accepted that no single assay will fully replace the rabbit Draize eye test.

2.3.1.3. External collaboration. Equally important to achieve validated *in vitro* methods is collaboration between industry, academia, external scientific organisations and regulators.

- COLIPA is working with ECVAM by active mutual participation in both COLIPA and ECVAM Eye Irritation Task Forces.
- COLIPA is providing ECVAM with support for post hoc statistical analysis of current *in vitro* methods and development of approaches for integrated analysis to define batteries of *in vitro* assays via the funding of an independent biostatistician. This latter activity is further discussed in Section 2.3.3 below.

2.3.1.4. Research programme. Building on the experience of earlier validation studies and scientific workshops the research programme is focused on identification of *in vitro* endpoints related to the dynamics of injury and recovery that are more predictive for *in vivo* eye irritation effects that occur in humans. This will enable the development of prediction models for pre-validation of new or improved *in vitro* methods that would proceed to formal validation.

The original research programme, which is now completed, was composed of three integrated research projects: (1) development of an *in vitro* model of excised corneas maintained in culture to allow observation of injury/recovery after chemical exposure to investigate whether kinetics/patterns of change in physiological function and signals of injury released from the cornea *in vitro* can predict a chemical's potential to damage the eye with a focus on recovery; (2) development of sequentially built bioengineered 3-D multi-layer corneal constructs consisting of epithelium, stroma and endothelium to better understand underlying mechanisms of action to enable identification of endpoints related to magnitude of injury and quality of repair in human immortalised cells and 3-D human conjunctival and corneal constructs; and (3) a preliminary genomics project using a pattern recognition approach to identify new endpoints for injury and repair that builds on corneal models for potential use in current/future *in vitro* assays.

The goal of the research programme is to have available a model that measures depth of injury and recovery. As such, the ongoing work is focussed on continued development of multilayer corneal models such as isolated eyes, isolated corneas and 3-D bioengineered corneal constructs and incorporation of evaluation parameters that measures depth of injury as this relates to extent of recovery.

2.3.2. In silico approaches

Eye irritation is a difficult endpoint to model *in silico* because of the complexity of the biological mechanisms that may be involved (Maurer et al., 2002; Jester, 2006). Most approaches have therefore modelled eye irritation resulting from physical effects, such as penetration of the ocular tissues or corrosion. The most commonly used approaches have focused on the development of: (a) traditional quantitative models (in which a measure of eye irritation potency is predicted); (b) classification models (in which a classification of eye irritation potential is predicted); (c) simulation of molecule-membrane interactions; (d) neural network models in which non-linear QSAR modelling techniques are employed; and e) expert system approaches (e.g., TOPKAT, the BfR rules for the prediction of skin irritation (BfR), Derek (LHASA Ltd) and TOPKAT). A Decision Support System (DSS) for the prediction of several toxicological endpoints has been developed by the German Bundesinstitut für Risikobewertung (BfR). With regard to the prediction of eye irritation/corrosion, the DSS contains 31 physicochemical exclusion rules based on molecular weight, octanol–water partition coefficient, lipid/aqueous solubility and melting point, and 27 inclusion rules that define structural alerts potentially responsible for eye irritation and/or corrosion. A substructure is considered "reactive" based on the classification and labelling of the majority of its molecules (e.g., aliphatic monoalcohols) (Gerner et al., 2005; Tsakovska et al., 2007). This system was submitted to ECVAM for consideration to enter the validation process and is currently under review.

2.3.3. External validation oriented activities

ECVAM organised in 2005 a workshop in which experts from academia, industry and regulatory authorities developed an approach that uses *in vitro* assays in combination along with an understanding of physicochemical parameters to evaluate the eye irritation of a substance. This strategy is called the Bottom–Up and Top–Down approach and was developed to be applicable to chemicals in general (Bottom–Up and Top–Down approach: Eye irritation testing strategy to reduce and replace *in vivo* studies. Scott et al., submitted for publication). It relies, as a first step, on using physicochemical parameters to predict whether a substance would be expected to be an eye irritant or not. This decision drives the choice of the first *in vitro* assay to be conducted. Substances predicted to be of no or low eye irritation potential would first be tested in an *in vitro* assay most suited to the detection of non or mild irritants whilst those predicted to be severe eye irritants would first be tested in assays most suited for that purpose e.g., BCOP, ICE. This first *in vitro* assay is then followed by a second *in vitro* assay to confirm irritancy status of substances identified as not severe in the Top/Down testing scenario and those identified as irritant in the Bottom/Up testing scenario. It is the combination of the two *in vitro* assays that can then be used to finally clarify the eye irritation potential and if needed regulatory classification of a substance (GHS category 1 and 2 (Globally Harmonised System; a new legislation, recently approved to be implemented in the EU (EU, 2008)); R41, R36, NC according to the current EU classification system). In order to implement this approach, there is a need to have available a number of validated *in vitro* assays and a method of statistical analysis to determine the confidence of predictive capacity that can be derived from combinations of *in vitro* assays. A number of activities are ongoing to address this in terms of: (1) new (RhT assays validation study) and (2) retrospective (currently available cell function and cytotoxicity assays (Cytosensor, Red Blood Cell (RBC), Neutral Red Release (NRR), Fluorescein Leakage (FL) review) validation activities of *in vitro* assays. These activities as they relate to the new and retrospective validation activities for *in vitro* assays are listed in Table 2. Furthermore development of the statistical tools to support this integrated analysis will need to occur.

Along similar lines, the European Partnership on Alternative Approaches to Animal Testing (EPAA) and ECVAM, in a joint effort, held a workshop (EPAA, 2008) on integrated testing strategies which started to address the validation of integrated testing strategies and included the application of integrated analysis for definition of combinations of *in vitro* assays.

2.3.4. Other approaches

Another approach that has been suggested is use of a "Threshold of Toxicological Concern-like (TTC-like) approach". This refers to universal exposure thresholds of chemical exposure in general similar to the TTC approaches developed for systemic effects (Kroes et al., 2004, 2007). Whereas thresholds of concern are evaluated by safety assessors for specific substances and products on a routine basis, a universal threshold concept has not been systematically explored for eye irritation to date.

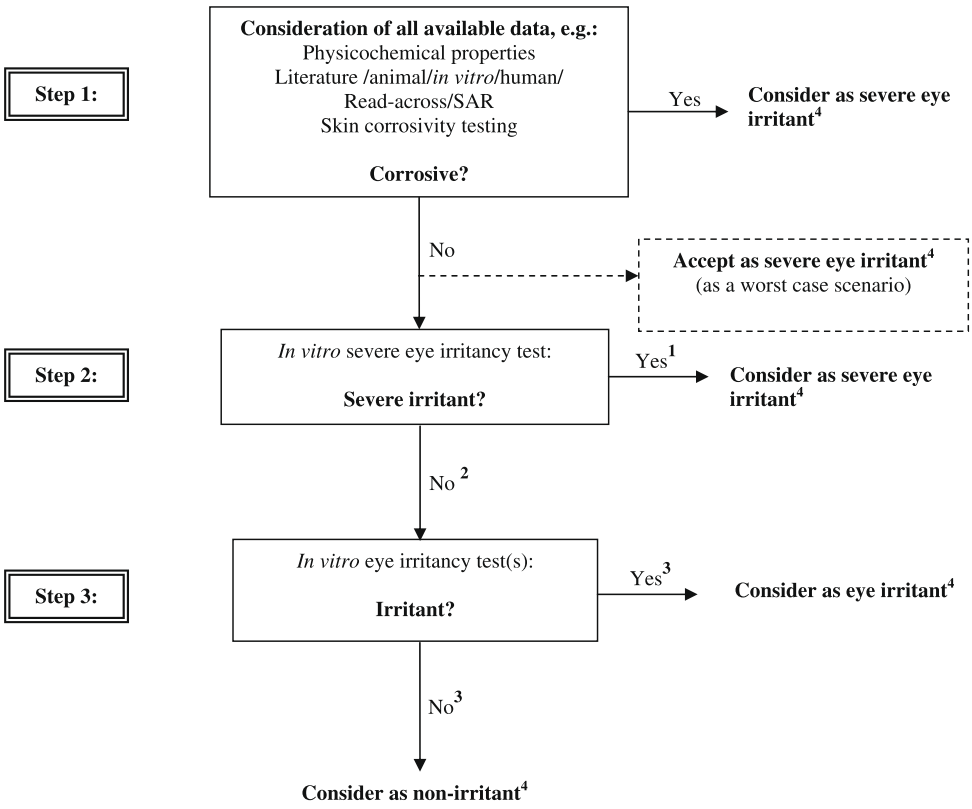
3. A tiered approach for eye irritation assessment

3.1. Decision tree for hazard assessment

A decision tree for eye irritation hazard assessment is outlined in Fig. 1. This is a tiered iterative approach based on a number of steps. It should be noted that decisions in this tiered approach take into account both *in vitro* assays that already have regulatory acceptance and those that are described in the scientific literature and based on industry experience as being "suitable for purpose". Such industry experience is typically based on individual company use of the *in vitro* assay with specific classes of chemicals that provide confidence for its predictive capacity relative to the substance under evaluation.

The different steps in the decision tree for hazard identification are as follows:

Step 1: Perform a WoE evaluation of all available information, including for example physicochemical properties and 'read-across' to other structurally similar chemicals with known eye irritation potential to answer the question "Is this substance likely to be corrosive to skin?". If the answer to this question is "yes", the substance is considered as corrosive and by default, judged as a severe eye irritant. If the answer to this question is "no", either consider it as a severe irritant as a worst case assumption, though not confirmed by testing, or proceed to Step 2.



To note: Decisions in this tiered approach take into account *in vitro* assays that already have regulatory acceptance as well as those that are described in the scientific literature as being suitable and/or for which industry experience is available.

[1] BCOP, ICE accepted by EU and USA regulatory authorities to identify severe irritants; IRE and HET-CAM also accepted by EU regulatory authorities for this purpose.

[2]Accepted on the basis of current practices and literature. Negative results not regulatory accepted, but planned ICCVAM activity for data analysis to identify levels of irritancy lower than severe irritancy for BCOP and ICE.

[3]*In vitro* test(s) to further position irritation potential lower than severe not currently accepted by EU. Validation by ECVAM in progress (e.g. EpiOcular™ and SkinEthic HCE™ assays).

[4]Classification systems (GHS and EU): Cat 1 (GHS) and R41 (EU) = severe irritant; Cat 2A and Cat 2B (GHS) and R36 (EU) = irritant; NC (GHS and EU) = Not classified

Fig. 1. Decision tree approach proposed to evaluate ingredients for hazard identification using alternative eye irritation assays.

Step 2: Conduct an organotypic assay listed in Table 1 to determine whether the substance is a severe eye irritant to answer the question "Is the severe eye irritancy of the substance confirmed?" BCOP and ICE are organotypic assays for which both external validation status and regulatory acceptance are already officially available for their use to positively identify severe eye irritants. Other organotypic models, the HET-CAM and IRE assays, are also accepted by EU authorities for the classification of severe irritants. If the outcome of the organotypic assay indicates that the substance is a severe eye irritant, the result of Step 1 is confirmed. If the organotypic assay does not indicate severe eye irritancy of the substance, in-house company experience is needed to support prediction of irritancy levels of less than severe in order to move to Step 3. It is important to consider the expected mode of action of the test material. When evaluating the routine endpoints in the organotypic assays, histological observations can improve the interpretation.

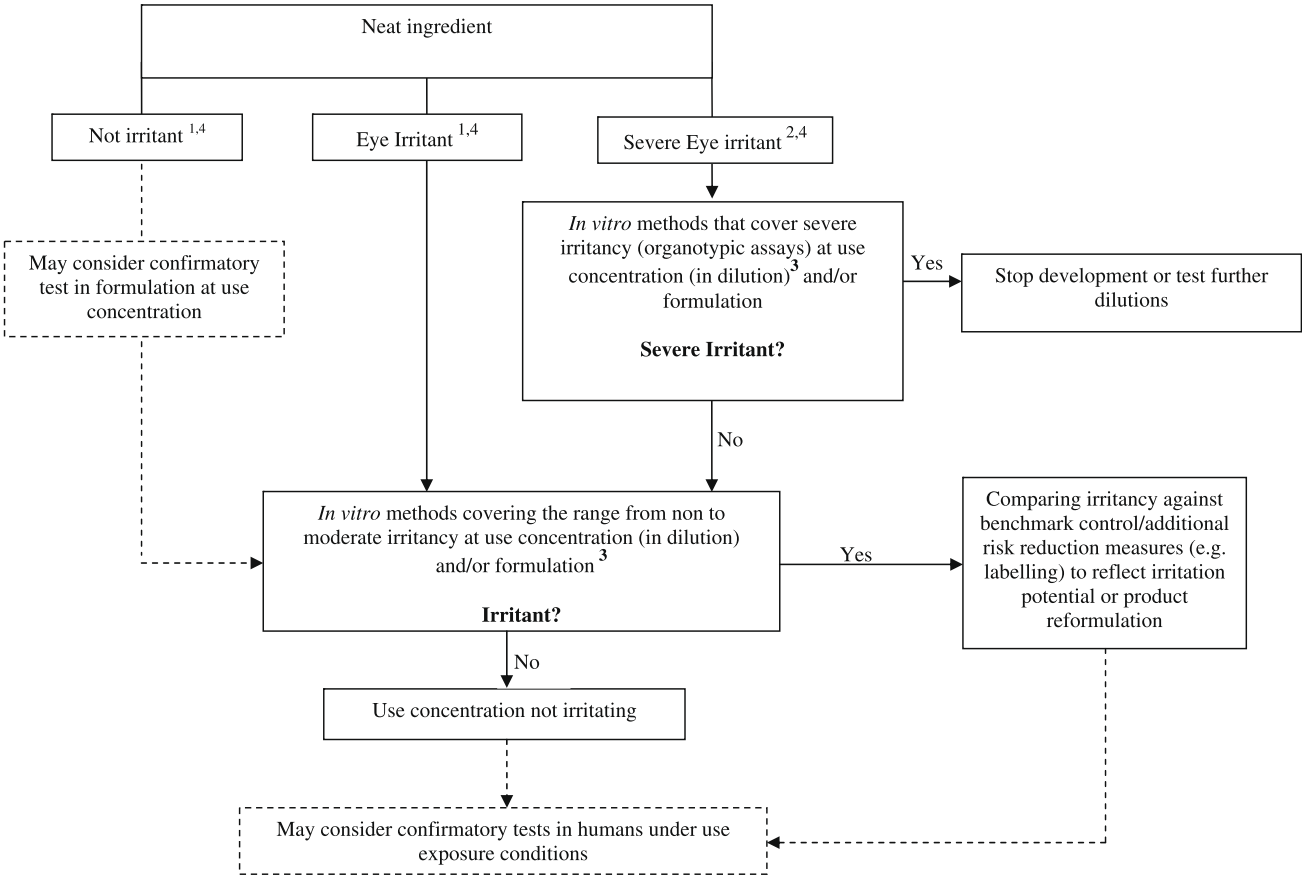
Step 3: Conduct an *in vitro* assay for eye irritation to answer the question "Is the substance an eye irritant (less than severe)?". The choice of assay(s) need to take into account its applicability domain and requires experience with the chemical class to be evaluated. The outcome of assay(s) in the WoE approach will indicate whether the substance is considered an eye irritant or not irritating to the eye.

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 and/or those that industry has experience with (in-house qualification). Regulatory acceptance information is included in the footnotes of Fig. 1.

Tables 1 and 2 provide an overview of *in vitro* eye irritation assays and their application based on peer-reviewed literature whilst Table 1 summarises the regulatory status of several of the assays.

3.2. Decision tree for safety assessment

A decision tree for safety assessment of ingredients using alternative eye irritation models is outlined in Fig. 2. As for hazard iden-



To note: Decisions in this tiered approach take into account *in vitro* assays that already have regulatory acceptance as well as those that are described in the scientific literature as being suitable and/or for which industry experience is available.

[1]*In vitro* tests to further position irritation potential lower than severe (i.e. irritant/not classified) not currently accepted by EU. Validation by ECVAM in progress but not expected to be completed before March 2009 (e.g. HCE assay)

[2]Negative result not regulatory accepted, but ECVAM/ICCVAM validation planned for BCOP, ICE

[3]*In vitro* tests to further position use concentrations of chemicals in dilutions (i.e. for identification of severe/irritant/not classified) not currently accepted by authorities

[4]Classification systems (GHS and EU): Cat 1 (GHS) and R41 (EU) = severe irritant; Cat 2A and Cat 2B (GHS) and R36 (EU) = irritant; NC (GHS and EU) = Not classified

Fig. 2. Decision tree approach proposed to evaluate ingredients identified as non-irritant, irritant or severe irritant (in Fig. 1) for safety assessment using alternative eye irritation models.

tification, the safety assessment decision tree is based on a WoE approach. The starting point of the safety assessment for ingredients in cosmetic products is hazard identification as presented in the decision tree detailed in Fig. 1.

It should be noted that, as for hazard identification, decisions in this tiered approach for safety assessment of substances take into account both *in vitro* assays that already have regulatory acceptance and those that are described in the scientific literature and based on industry experience as being suitable for purpose. Such in-house experience is typically based on individual company use of the *in vitro* assay with specific classes of chemicals that provide confidence for its predictive capacity relative to the material under evaluation. Very often, *in vitro* tests are performed with an ingredient in the context of a formulation in order not only to obtain information on the neat ingredient, but at the same time on the irritant potential of the actual composition of ingredients. This involves use of a comparative approach in which benchmark products are included in the test.

The different steps in the decision tree for safety assessment are as follows:

3.2.1. If the substance is identified as a severe eye irritant (Section 3.1)

Step 1: Conduct an organotypic *in vitro* eye irritation assay listed in Table 1 on the diluted substance to answer the question "Is the substance severely irritant at use concentration?". If the answer is "yes", use of this substance at this specific concentration in cosmetic products may not be possible. A severe irritant can also be tested at usage concentration in a formulation using organotypic *in vitro* eye irritation assay(s) to address that dilution and formulation effects may decrease the potential for eye irritation or confirm that the substance in formulation remains a severe eye irritant. Formulations need to have an acceptably low eye irri-

tation profile to ensure product safety in use and through accidental exposure.

If the diluted chemical does not cause severe eye irritation, proceed to Step 2.

Step 2: Conduct further *in vitro* tests covering the irritancy range from non- to moderate irritant to answer the question "Is the substance irritant or non-irritant at usage concentration?". This may take the form of testing diluted substance alone or at usage concentration in formulation. The choice of assay(s) needs to take into account its applicability and requires experience with the chemical class/formulation category to be evaluated. The outcome of assay(s) in the WoE approach will indicate whether the diluted substance/substance at usage concentration in formulation is considered an eye irritant or not irritating to the eye. If the answer to this question is "yes" a comparison is made with benchmark substances/formulations to determine acceptability for continued development/marketing and whether additional eye irritant labelling measures are required, or re-formulation needed. If the answer is "no", no further evaluation is required.

As mentioned above, 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 for purpose (see Tables 1 and 2 for additional information on assay application and regulatory status of different assays).

Once the eye irritation profile of the substance at use concentration is established, in some instances, it is desirable to obtain more information about formulations that are used repeatedly around the eye area. In these circumstances, confirmatory human testing of such formulations may be considered provided that a safety assessment is available that supports this level of human exposure.

If the result indicates that the substance is irritant (less than severe) (*Section 3.1*): *Step 1:* Conduct *in vitro* tests covering the irritancy range from non- to moderate irritant to answer the question "Is the substance irritant or not irritant at usage concentration?". The outcomes from this test are handled in the same way as those from Step 2 for substances identified as severe.

If the result indicates that the substance is not an irritant (*Section 3.1*): if additional information is desirable, *in vitro* eye irritation assays that cover the range non- to moderate irritant may be considered for testing the substance at usage concentration in formulation. This could be considered if formulation effects could be suspected to alter the irritancy profile of the substance at usage concentration. The *in vitro* assays for this purpose are chosen on the same basis as described above in Step 2 for severe irritants. The outcome of *in vitro* assays will confirm the non-irritancy of

Table 3
Use examples based on information from 11 companies.

Assay(s)	Ingredient	Products (containing specific ingredients)
HET-CAM	Various ingredients[8] (not routinely performed, but data on various substances is available and has been used in validation studies)[3]	Used mainly to assess surfactant-based cosmetic products. However, other categories of ingredients are also contained in such formulations and then also covered by the assessment[3] Various types of formulations[5] Rarely used for formulations[9]
HET-CAM + RBC (1 h)	Botanicals (for several extracts like propylene glycol and glycerol HET-CAM is over-predictive and therefore not suitable), polymers, silicones (RBC depending on solubility), natural and mineral oils (RBC depending on solubility), UV filters (RBC depending on solubility), emulsifiers[2]	Alkalis, botanicals (for several extracts e.g., propylene glycol and glycerol HET-CAM is over-predictive and therefore not suitable), pigments, polymers, silicones, natural and mineral oils, UV filters, solvents (not for higher concentrations), emulsifiers[2]
HET-CAM + NRR	Botanicals, polymers, silicones, natural and mineral oils, solvents (up to 10%), surfactants (up to 10%), emulsifiers, perfume oils. For some ingredients containing more than 10% of surfactants, NRR test is over-predictive, in these specific cases the HCE model is used[4]	Perfume oils; rinse-off formulations. When discrepancies are observed the two methods, HCE is used[4] Various formulations[8]
HET-CAM + HCE		Surfactants[4]
HET-	CAM + HCE + BCOP	Silicones[6]
BCOP		Highly alkaline formulations[1] Used mainly to assess surfactant-based cosmetic products. However, other categories of ingredients are also contained in such formulations and then also covered by the assessment[3]
BCOP + CAMVA		Various types of formulations[5]
EpiOcular™ (MatTek)	Pigments, silicones[11]	Botanicals, colored formulas (hair dyes NA), pigments, polymers, preservatives, silicones, solvents, surfactants (if not highly alkaline)[1] EpiOcular is used for personal care products that are expected to be non-irritating to mild[3] Botanicals, pigments, silicones, UV filters, polymers, emulsifiers, perfume oils, preservatives (face creams and colour cosmetics); leave-on formulations, other formulations (hair sprays, deodorants, antiperspirants)[11] Surfactant-based formulations (benchmark approach for baby and adult shampoos)[10]
EpiOcular™ + ICE	Botanicals; [colorants (primarily ICE, may include EpiOcular™)][11]	[Colorants (primarily ICE, may include EpiOcular™)][11]
EpiOcular™ + RBC	Surfactants (10 min-RBC) and in special cases EpiOcular)[2]	Solvents (for higher solvent concentrations), Surfactants (10 min-RBC and in special cases EpiOcular)[2]
ICE	Alkalis, colorants (primarily ICE, may include EpiOcular)[11]	Alkalis, colorants (primarily ICE, may include EpiOcular)[11]
ICE + cytosensor	Surfactants[11]	Surfactants, preservatives (surfactant-based formulations); rinse-off formulations[11]
NRR + HCE		Leave-on formulations[4]
IRE	Various types of ingredients, especially as screen for severe irritants; only occasionally for safety assessment, but routinely for occupational hazard assessment[7]	Various types of formulations[10]
HCE (3-D cornea model, SkinEthic™)	Solvents (>10%), surfactants (>10%)[4] Most ingredients; silicones with limitation (combination with HET-CAM + BCOP to enhance sensitivity/predictive performance)[6] Various types of ingredients; only occasionally for safety assessment, but routinely for occupational hazard assessment[7]	Mascara: due to the galenic of this kind of product, only the HCE model is suitable[4]
TEP		Various types of formulations (Surfactant-based or generally used in the eye area)[9]

The suffixed number after each entry identifies an individual company's response.

the substance at use concentration in formulation or that formulation effects have increased the irritation potential.

Again, as described above, confirmatory human testing can be considered as necessary once the safety assessment for eye irritation is complete.

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

Eleven manufacturers of cosmetic products, including major companies, gave feedback on their use of *in vitro* assays to give an overview of which assays are typically used for safety assessment. Despite a lack of formally validated and accepted assays beyond the organotypic assays for specific and limited regulatory purposes, multiple *in vitro* assays are routinely used for this purpose. Table 3 provides examples of assays or combinations of assays that are used to evaluate the eye irritation potential of cosmetic ingredients or products.

The approach typically used by companies to define the assay or combination of assays to be used for evaluation of eye irritation is based on combining knowledge/information from several different sources. These include:

- Information for individual assays on "suitability for purpose" from:
- Peer-reviewed scientific literature and previous validation/evaluation studies that define the predictive capacity of an assay in terms of range of irritancy and/or chemical class. Such information would also include any limitations that are known for the assay (e.g., chemical class, physical form). Uses of *in vitro* assays for specific product/ingredient types are indicated in Tables 1 and 2.
- In-house experience gained from use of the assay(s) that may relate to peer-reviewed literature and previous validation/evaluation studies. Such experience is typically in the context of formulation testing since it is the finished product to which a consumer is exposed. This is based on a comparative toxicology approach in which appropriate benchmark products are also included. Collation of these data over a period of time increases the reliability to support practical application of an assay/combination of assays tailored to a company's product ingredients. Uses of *in vitro* assays for specific product/ingredient types are indicated in Tables 1–3.
- Information on the proposed mechanism by which the ingredient/product causes eye irritation. It was defined earlier that mechanisms of injury causing acute effects include membrane lysis, protein coagulation, saponification and action on macromolecules (Maurer et al., 2002; Jester, 2006). Examples of substances that act by these mechanisms are surfactants (membrane lysis), acids, alkalis and organic solvents (coagulation), alkalis (saponification) and bleaches/peroxides (action on macromolecules). An example here would be the use of organotypic assays for evaluation of surfactant-based formulations.
- Information on the design and properties of the *in vitro* assay to enable choice of assay(s) based on mechanistic understanding of eye irritation. This relates to understanding of:
  - The *in vivo* endpoint that the *in vitro* assay is designed to model.
  - The structure of the *in vitro* model for example whether it is a single monolayer of cells (Fluorescein Leakage); a stratified epithelium (RhT), a complete cornea (BCOP), whole eye (ICE).

Current knowledge supports that area/depth of corneal injury are principal factors in early responses and eventual repair after accidental eye exposure and that physiological changes and inflammation are secondary responses. Slight irritants tend to affect only the superficial corneal epithelium, mild and moderate irritants affect epithelium and superficial stroma whilst highly moderate and severe irritants affect deeper layers of the stroma (and possibly the endothelium). On this principle, *in vitro* models that include a stroma (e.g., BCOP, ICE) are more likely to be able to detect severe irritants whilst those based on epithelium only (RhT assays) are likely to predict better at the lower to middle range of irritancy.

As such, it is through a collation of information as detailed above on the specific *in vitro* assays, experience with conduct of the assays, and use of appropriate benchmark controls that allows companies to choose combinations of assays that are targeted, complementary to each other and appropriate for the evaluation of eye irritation for specific classes of ingredients/product types.

5. Future needs

5.1. Validated and regulatory accepted in vitro eye irritation assays

Formal validation of *in vitro* assays for their specific domains of applicability and acceptance by the regulatory authorities in order to predict across the range of eye irritancy for different chemical classes.

5.2. Optimization of current assays

Continued development of current assays using different evaluation endpoints to extend their domain of applicability.

5.3. Dose–response considerations

Investigation of current or newly developed methods to find more sensitive evaluation parameters to enable better discrimination at the low irritancy range (mild and non-irritants).

5.4. Integrated analysis

Today, companies assess the eye irritation potential of ingredients and formulations using specific combinations of *in vitro* assays that are based on experience. This approach should be further developed and refined through use of statistical tools such as integrated analysis that objectively defines predictive performance of test combinations. It is anticipated that as more assays are formally validated, they will be incorporated into *in vitro* batteries using integrated analysis, a process which itself may be subject to official acceptance criteria.

5.5. Focus on mechanistically-based assays

Development up to scientific validation of *in vitro* assays that are based on a better understanding of mechanisms of eye irritation, especially with focus on recovery.

5.6. (Q)SAR validation

Further evaluation of emerging (Q)SAR models for eye irritation.

6. Conclusions

There are a number of alternative models available and already established within the cosmetics industry which can be used to assess the potential of cosmetic ingredients to cause eye irritation, although none are formally validated as full stand-alone replace-

ments for the rabbit Draize eye test. Some assays (organotypic assays) are accepted by regulatory authorities for specific and limited purposes. Despite a number of extensive validation studies, the acceptance of *in vitro* assays by regulatory bodies beyond those accepted today will require more data and review.

Regulatory accepted and non-validated *in vitro* assays are used for the evaluation of eye irritation of ingredients used in cosmetic products. Confidence in the evaluation of eye irritation potential is increased by using combinations of assays since they may provide information over the entire range of irritancy (from non-irritant to severe) for different classes of chemicals. A combination of the regulatory accepted and non-validated assays together with all other available information in a tiered approach which is based on a WoE evaluation in each step provides a reasonable framework for the evaluation of eye irritation of ingredients used in cosmetic products. General acceptance of using such an approach is a pre-requisite for replacing animal studies.

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