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STREAMLINED SUMMARY DOCUMENT SUPPORTING OECD TEST GUIDELINE 438 ON THE ISOLATED CHICKEN EYE FOR EYE IRRITATION/CORROSION

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2013

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FOREWORD

This streamlined summary document (SSD) was developed to provide summary information in support of OECD Test Guideline 438 on the Isolated Chicken Eye Test Method addressing the endpoint eye irritation/corrosion. This SSD was developed by a Secretariat consultant and submitted to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in March 2013, together with the updated version of TG 438 (originally adopted in 2009). The SSD provides useful and more detailed information than is otherwise available from the Test Guideline itself on: 1) the scientific basis of the test method, 2) the identified limitations, weaknesses and strengths, 3) the applicability domain, 4) the sensitivity, specificity and accuracy, and 5) the within-laboratory and between-laboratory reproducibility of the method.

The SSD was approved by the WNT with a few changes to paragraph 11, including additional references 22, 23, 24 and 25, on 30 April 2013.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 14 June, 2013.

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

STREAMLINED SUMMARY DOCUMENT

Description of applicability domain and performance, based on the retrospective validation studies and their revisions, of the Isolated Chicken Eye (ICE) Test Method (Test Guideline 438) for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage

INTRODUCTION AND BACKGROUND

The 2003-2006 Validation Studies

1. Between 2003 and 2006, a retrospective evaluation was carried out concerning the validation status of the Isolated Chicken Eye (ICE) test method for identifying chemicals (substances and mixtures) inducing serious eye damage ("ocular corrosives and severe irritants"), *i.e.*, its usefulness and limitations for initiating a Top-Down approach (1). This evaluation, counting with a total of 175 chemicals, was performed by the US-Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the US-National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), in collaboration with the EU-European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM). For a full description, see the Background Review Document (BRD) (2) and the ICCVAM Test Method Evaluation Report (TMER) (3).

2. The study aimed at characterising the reproducibility and predictive capacity of the ICE for the following classification systems: UN GHS (Category 1) (4), US EPA (Category I) (5) and EU DSD (R41) (6) (the EU CLP classification system (7) based on UN GHS had not yet been adopted at that time). On the basis of all collected data and information the ICE was considered as scientifically valid (reliable and relevant) for identifying chemicals inducing serious eye damage (*i.e.*, to initiate a Top-Down approach (1)) and was recommended for regulatory hazard classification and labelling purposes. Chemicals inducing serious eye damage are defined as those that produce tissue damage in the eye, or serious physical decay of vision, following application to the anterior surface of the eye in the *in vivo* Draize rabbit eye test (8), which is not (or not expected to be) fully reversible within 21 days of application. Following these recommendations, the OECD officially adopted the ICE test method as OECD Test Guideline (TG) 438 for identifying chemicals inducing serious eye damage in September 2009 (9).

The 2006-2009 Validation Studies

3. NICEATM/ICCVAM, in collaboration with ECVAM and JaCVAM, further evaluated between 2006 and 2009 the usefulness and limitations of the ICE test method for the additional identification of chemicals not causing sufficient effects on the eye to require hazard classification and labelling according to the UN GHS (4), US EPA (5), US FHSA and EU DSD (6) classification systems (the EU CLP classification system (7) based on UN GHS had not been adopted at that time), *i.e.*, its usefulness and limitations for initiating a Bottom-Up approach (1). The ICE validation database remained unchanged and comprised 175 chemicals (90 substances and 85 mixtures) collected from five individual studies (Prinsen and Koëter 1993 (10), Balls et al. 1995 (11), Prinsen 1996 (12), Prinsen 2000 (13) and Prinsen 2005 (14)), which were used to determine the predictive capacity of the ICE test method (ICCVAM BRD from April 2009 (15)).

4. In May 2009, NICEATM/ICCVAM convened an independent international scientific peer review panel (PRP) on alternative ocular safety testing methods, composed of members from EU, USA, Japan and

Canada. The PRP maintained the original recommendation for using the ICE for classification of serious eye damage (16). At that stage however, no further recommendations were made for an expansion of the ICE applicability domain to also include other classification categories, and in particular the identification of non-classified chemicals (16). This was due to the false negatives rates (6% or 4/62 for the UN GHS classification system) and the fact that amongst the false negatives there was one substance was classified as GHS Cat 1 based on Draize rabbit eye test data (16).

Revisions of the Validation Dataset

5. On the basis of revisions of the validation dataset carried out in 2012 it was found that the individual *in vitro* and *in vivo* classifications of a number of chemicals deserved further considerations. In particular discrepancies were found in the final *in vivo* and *in vitro* classifications for a number of chemicals in the ICCVAM BRD from April 2009 (15) which had an impact on the number of false negative chemicals (1 out of 73 false negative instead of 4 out of 62; see Appendixes 1, 2 and 3). In addition, it was felt important to recognize the limitations of the *in vivo* Draize rabbit eye irritation test and their implications for validation purposes (17). Some of the drawbacks of the Draize rabbit eye test referred in literature include (see extract from Eskes (18) in Appendix 3 for details):

- The fact that the *in vivo* rabbit eye irritation/corrosion test has no standardized exposure regimen, so that the duration of exposure of the test substance with the rabbit eyes remains unknown and can vary from a few minutes to several hours. In addition, for solids and sticky chemicals it is unclear how much of the compound (solid, paste or liquid) stays in contact with the eye (19);
- The limited reproducibility of the Draize rabbit eye test method;
- The subjectivity in the allocation of the rabbit ocular tissue scores;
- The type of exposure which does not reflect a potential human accidental exposure;
- The differences in physiology and sensitivity to tested chemicals between rabbit and human eyes;
- Ethical issues and the fact that the Draize test can be very painful to the rabbits.

Adoption of TG 438 for the Identification of Chemicals Not Classified for Eye Irritation or Serious Eye Damage

6. In April 2012, following a proposal from the Netherlands and the European Commission, a project for updating TG 438 (9) was included in the work plan of the OECD Test Guidelines Programme. The aim of the project was, taking into account the review of the individual *in vivo* and *in vitro* data, to reassess the performances of ICE and address a possible update of TG 438 to allow its use also for the identification of chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system. An initial re-evaluation of the *in vitro* and *in vivo* ICE dataset was carried out by the Netherlands, followed by the preparation of an Issue Paper and its addendum by a consultant to the OECD Secretariat with the aim to review existing ICE data and make a recommendation on the use of TG 438 for the identification of chemicals not requiring classification for eye irritation or serious eye damage (Appendixes 3 and 4). The ICE Issue Paper and its addendum reviewed both the ICE data presented in the ICCVAM-NICEATM ICE BRDs (2) (15) as well as the evaluation carried out by The Netherlands in the first draft version of this SSD (17). The Issue Paper and its addendum were discussed by the eye irritation/corrosion expert group at an OECD expert meeting held on 6-7 December 2012. The present SSD represents a compilation of all relevant data on the ICE test method evaluation and takes into account all comments received at and after the eye irritation expert meeting, as well as the comments received from the two commenting rounds on the proposed revised TG 438.

7. In 2013, the OECD approved the updated version of TG 438 allowing the use of ICE for identifying chemicals inducing serious eye damage as well as for identifying chemicals not requiring classification for eye irritation or serious eye damage.

SCIENTIFIC BASIS FOR THE ICE TEST METHOD

8. The ICE test method (TG 438) is an organotypic model that provides short-term maintenance of the chicken eye *in vitro*. Damage to the cornea is assessed by determination of corneal swelling, corneal opacity, and fluorescein retention in a test that typically takes less than 8 hours to perform. Analysis of corneal swelling provides for a quantitative assessment, whereas corneal opacity and fluorescein retention involve a qualitative assessment based nevertheless on slit-lamp observations. Each endpoint is then either converted into a quantitative score or assigned a qualitative categorization (ICE Classes) which are then combined together and used to assign an *in vitro* ocular hazard classification (9) (20).

Data Interpretation Procedures

9. If the criteria used to attribute the ICE classes (I to IV) for the three endpoints (corneal swelling, corneal opacity and fluorescein retention) remained unchanged, the Data Interpretation Procedure (DIP) proposed by ICCVAM and in the OECD Guidance Document 160 (15) (20) differed slightly from the one proposed by The Netherlands (see Appendix 3). Following consultation of the OECD Expert Group on Eye Irritation, it was recommended to make use of the DIP proposed by ICCVAM and by Guidance Document 160 (15) (20) complemented with some effects proposed by The Netherlands as described in Table 1.

Table 1. Data Interpretation Procedures applied for the ICE test method for identifying *i)* chemicals inducing serious eye damage and *ii)* chemicals not requiring classification for eye irritation or serious eye damage.

UN GHS Classification	Combinations of the 3 Endpoints*
No Category	3 x I 2 x I, 1 x II
No prediction can be made	Other combinations
Category 1	3 x IV 2 x IV, 1 x III 2 x IV, 1 x II** 2 x IV, 1 x I** Corneal opacity ≥ 3 at 30 min (in at least 2 eyes) Corneal opacity = 4 at any time point (in at least 2 eyes) Severe loosening of the epithelium (in at least 1 eye)

* Based on the criteria proposed in the original TG 438 (9) and in the Guidance Document 160 (20)

**Combinations less likely to occur.

Comparison of the ICE Test Method with the In Vivo Rabbit Eye Test Method

10. In contrast to ICE, the *in vivo* rabbit eye test involves only qualitative evaluations based mainly on visual observations of the severity of adverse effects on the cornea, the iris, and the conjunctiva, as well as the reversibility of any ocular effects detected at selected intervals up to 21 days after exposure. In ICE, liquids and solids are typically tested undiluted and are applied to evenly cover the entire surface of the cornea. In the *in vivo* rabbit eye test, liquid and solid test substances are also tested usually undiluted, however they are applied to the conjunctival sac of the rabbit eyes. Because rabbits blink and/or tear, exposure of the cornea to the test substance will be affected by these factors in terms of coverage or

duration. The neurogenic components that drive tear film production are not present in the ICE. When compared with an *in vivo* rabbit eye study, application of a test substance in the absence of this protective barrier might be expected to cause an increase in false positive outcomes. One of the conclusions from a workshop on mechanisms of eye irritation highlighted the need for additional research on the impact of chemicals on tear film and the consequences of tear film disruption. However, for some test substances (*e.g.,* solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. Thus, the ICE test method differs from the *in vivo* rabbit eye test method in the following significant ways:

- The ICE evaluates only corneal effects and does not assess effects on the iris and the conjunctiva as performed in the *in vivo* rabbit eye test. Measurements are performed quantitatively and qualitatively with the help of a slit-lamp in the ICE assay, while they are assessed only qualitatively based mainly on visual observations in the *in vivo* rabbit eye test.
- Corneal exposure conditions, including test substance concentration and exposure duration, are well defined in the ICE assay, whereas subject to potentially large variations *in vivo* due to the ill-defined exposure conditions, blink response and natural tearing of the eye in a live animal. Moreover, based on the unrealistic accidental *in vivo* exposure conditions, solids may lead to variable and extreme responses in the *in vivo* Draize eye irritation test, which may not reflect their true irritation potential in humans (19).
- The observation period of the ICE assay is typically of 4 hours, whereas ocular effects are typically evaluated in the *in vivo* rabbit eye test for a minimum of 72 hours and can extended up to 21 days.
- Reversibility/irreversibility of corneal effects induced by a test substance cannot be observed in the ICE assay *per se*, but histological evaluation of the exposed eyes may provide additional information about the depth and type of injury that could aid predictions, as to whether damage is irreversible. It has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury may be used to identify some types of irreversible effects (21) although further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur.
- Protective mechanisms of the eye, such as tear production and blinking (e.g., against drying and infection), are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing. However, if regeneration of the tear film might be important for the *in vivo* healing process, it may play a minor role in the ICE test method where the initial damage is measured rather than recovery.
- The ICE assay does not account for systemic effects following ocular instillation that may be noted with the *in vivo* rabbit eye test (*e.g.,* toxicity or lethality as in the case of certain pesticides). However, these effects are typically predicted from other acute toxicity test methods, and may not be relevant for the many consumer products that are formulated with well characterized raw materials of known systemic toxicity.

IDENTIFIED LIMITATIONS, WEAKNESSES AND STRENGTHS

11. The potential shortcomings of the ICE test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) in e.g., a Top-Down approach, are based on the high false positive rate for alcohols and the high false negative rates for solids and surfactants, as observed in the 2003-2006 retrospective validation study (2) (3). When substances within these chemical and physical classes are excluded from the database, the accuracy of the ICE test method is substantially improved (2) (3) (see Table 6 below). However, since not all alcohols are over-predicted (4 out of 10) and some are correctly predicted as UN GHS Category 1, this organic functional group is not considered to be out of the applicability domain of the test method. Positive results obtained with alcohols should nevertheless be

interpreted cautiously due to potential over-prediction. Similarly, given the fact that *i)* some solids and surfactants are correctly predicted by the ICE test method as UN GHS Category 1, *ii)* that not all solids and surfactants are underpredicted (6 underpredictions out of 11 solids, and 6 out of 9 surfactants), and that *iii)* the underpredicted solids and surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (as none of the GHS Cat 1 solid and surfactant materials in the validation database are underpredicted as GHS non-classified), solids and surfactants are also not considered to be out of the applicability domain of the ICE test method. Evidence suggests that there is a certain probability that Cat 1 are predicted as Cat 2 due to the variability of individual animal responses within the same test (22). Although not based on the same dataset, the resulting probability seems to be in the same range as the BCOP/ICE under-prediction rate for identifying UN GHS Category 1. However, variability between laboratories can further contribute to the variability of *in vivo* responses (23)(24)(25). Quantitative estimates for such uncertainties, both for the in vivo tests and for their in vitro alternatives, should be considered in the future development of testing strategies for serious eye damage/eye irritation.

12. When used to identify chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system, in e.g., a Bottom-Up approach, anti-fouling organic solvent containing paint were found to risk under-prediction (1 out of 2 classified anti-fouling solvent containing paint was found to be under-predicted as non-classified). When chemicals within this product classes are excluded from the database, the accuracy of the ICE test method is only slightly improved (see Table 7 below). In addition, the only underpredicted material (TNO-94) was classified *in vivo* as GHS Cat 1 due to unusual effects, i.e., a residue of paint got attached to the cornea most probably caused by grooming/scratching of the eye by the rabbit and the type of exposure of the rabbit eyes (i.e., adding the paint in the conjunctival sac of the eye and holding the eye lids together). This was observed in only one animal, whereas the two other animals only showed GHS Cat 2-type effects (see Appendix 5). The OECD Expert Group decided to add a warning sentence for this category of materials, but not to exclude them from the applicability domain of the ICE test method for the following reasons: i) there was insufficient evidence to exclude those types of formulations (only two classified chemicals from this category), ii) the type of exposure to this material is unlikely to occur in humans, and iii) sticky materials present similar difficulties to test either *in vitro* and *in vivo*. Regarding the false positive rates, surfactants (5 out of 8) may risk overprediction. However due to *i)* the fact that not all surfactants were overpredicted, *ii)* that some surfactants were correctly predicted, and that *iii)* surfactants not predicted as GHS non-classified would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (2) (as none of the *in vivo* GHS non-classified chemicals (n=73) was overpredicted as GHS Cat. 1), surfactants are not considered to be out of the applicability domain of the ICE test method.

13. Although the ICE test takes into account some of the ocular effects evaluated in the *in vivo* test method and to some degree their severity, it does not consider conjunctival and iridal injuries. Nevertheless, the ICE directly addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification system (see Annex 6 of SSD for TG 437). In addition, Burton (26) found a direct relation between corneal swelling and the conjunctival reactions in a study with 600 rabbits and approximately 100 test substances. Prinsen (12) also reported a high correlation between conjunctival reactions and the endpoints assessed in the ICE test method after parallel testing of test substances *in vivo* and *in vitro*. Furthermore, although the reversibility of corneal lesions cannot be evaluated *per se* in the ICE test method it has been proposed, based on rabbit eye studies, that an assessment of the initial depth of corneal injury (*e.g.*, through histological evaluation) may be used to identify some types of irreversible effects (21). Finally, the ICE does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

14. The ICE test method is not recommended for the identification of test substances classified as irritating to eyes (UN GHS Category 2 or Category 2A) or test substances classified as mildly irritating to eyes (UN GHS Category 2B) due to the considerable number of UN GHS Category 1 chemicals underclassified as UN GHS Category 2, 2A or 2B and UN GHS No Category chemicals overclassifed as UN GHS Category 2, 2A or 2B. For this purpose, further testing with another suitable method may be required.

Table 2: Physicochemical properties and compatibility with the ICE

Physicochemical property	Is a material with this property compatible with the ICE assay?
Fixative	Yes
Solvent	Yes
Extreme pH	Yes
Gases	No
Liquids	Yes
Solid materials	Yes
Emulsions	Yes
Granular materials	Yes
Suspensions	Yes
Coloured materials	Yes
Diluted concentrations of chemicals	Yes
Highly viscous materials	Yes
Volatile materials	Yes
Reactive chemistries	Yes
Hydrophobic/lipophilic chemicals	Yes
Neat concentrations of chemicals	Yes

APPLICABILITY DOMAIN

15. The Test Guideline can be used for testing all types of substances and mixtures, provided there is no evidence that the method is not valid for the chemical tested**.**

Categories of Irritancy

16. Based on the conclusions of the 2003-2006 and 2006-2009 retrospective validation studies (3) (16), TG 438 was adopted in 2009 for classification of chemicals inducing serious eye damage (UN GHS Category 1). In addition, following the re-evaluations carried out in 2012 and their review by the OECD Expert Group on Eye Irritation, TG 438 was also approved in 2013 for the identification of chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category), under the UN GHS classification system.

Potential Role in an ITS

17. The ICE can be used as a validated *ex vivo* test method in a tiered testing approach as described in the addendum to TG 405 (8) with the purpose of identifying chemicals inducing serious eye damage (UN GHS Category 1), as well as chemicals not requiring classification for eye irritation or serious eye damage (UN GHS No Category). No published studies have been found at the current date, on the combination of ICE with other *in vitro* test methods in testing strategies for the prediction of ocular hazards.

Mode of Action (MoA)

18. An expert meeting held at EC-ECVAM in 2005 (1) recommended to expand the concept of defining the applicability domain as not only chemical classes, but also as a function of the mechanism of eye

irritation. The four identified MoA that were discussed included: (i) cell membrane lysis (breakdown of membrane integrity as might occur from exposure to membrane active materials, *e.g.,* surfactants), (ii) saponification (breakdown of lipids by alkaline action), (iii) coagulation (precipitation/denaturation of macromolecules, particularly protein, characteristic of acid, alkali, or organic solvent exposure), and (iv) actions on macromolecules (chemicals that react with cellular constituents/organelles that may or may not lead to overt lysis or coagulation, *e.g.*, peroxides, mustards and bleaches). The ICE test method addresses the three first MoAs. In addition, it may also address the fourth MoA (actions on macromolecules) when histopathological information is available.

Table 3: Summary of events involved in chemical-induced eye irritation *in vivo*

Events involved in chemical-induced eye irritation	Modelled by the ICE assay?
Chemical interaction with tear film	No
Chemical binding to the conjunctival epithelium	No
Adhesion molecules compromised	Yes
Corneal epithelial damage	Yes
Inhibition of receptor-mediated transport	Yes
Compromise of cell membrane integrity of upper corneal epithelium	Yes
Cell **membrane lysis** of all corneal epithelial layers	Yes
Hydration of corneal stroma	Yes
Cross-linking *of proteins in corneal stroma*	Yes
Erosion of corneal stroma	Yes
Cell damage to corneal epithelium and limbus	Yes
Dilation and increased lymphatic leakage from scleral vasculature	No
Stimulation of nerve endings, i.e*., enhanced blinking, tearing*	No
Erosion of nerve endings in corneal and sclera	No
Duration of the response, *i.e.*, length of time cell responses deteriorate. Duration of response covers the effects of **reactive chemicals**, which can cause **coagulation**, **saponification**, that are effects, which develop and increase over time.	Yes
Recovery from response, *i.e.*, length of time for cell responses to return to control levels	No

Chemical Classes

19. The ICE validation dataset comprised a total of 175 individual chemicals (90 substances and 85 mixtures) collected from Prinsen and Koëter 1993 (10), Balls et al. 1995 (11), Prinsen 1996 (12), Prinsen 2000 (13) and Prinsen 2005 (14) as described in the retrospective validation studies (2) (15). Tables 4 and 5 show the chemical and product classes representation within the ICE validation database. Although the single components from the mixtures used in the validation dataset could not be disclosed due to proprietary reasons they represented relevant to current commerce mixtures and formulations. In addition, details on the product categories and chemical classes of most of the tested mixtures and substances were available as described in the ICCVAM BRD from April 2009 (15). Out of the 175 chemicals, 85 (including mixtures) could not be assigned a specific chemical class and 23 (including substances) could not be assigned a specific product category. Detailed information, including chemical name, Chemical Abstracts Service Registry Number (CASRN), chemical class, product category, physical state, purity, concentration(s) tested, *in vivo* GHS classification, *in vitro* GHS classification, *in vitro* raw data, *in vitro* categories and literature reference using the chemical are provided in Appendix 1.

Table 4: Chemical classes tested in the ICE test method*.

Chemical Class	# of Chemicals	Chemical Class	# of Chemicals
Acetate	1	Inorganic Chloride Compound	1
Acid	5	Inorganic Salt	3
Acyl halide	1	Inorganic Silver / Nitrogen Compound	1
Alcohol	15	Ketone	4
Aldehyde	2	Lactone	1
Alkali	3	Lipid	1
Amide /Amidine	7	Nitrile	1
Amino acid	1	Nitro Compound	1
Boron compound	1	Not Classified	85
Carbohydrate	2	Onium Compound	8
Carboxylic acid	12	Organic Silicon Compound	2
Ester	10	Organic Sulfur Compound	3
Ether	1	Organometallic	2
Heterocyclic	9	Organophosphrous Compound	1
Hydrocarbon	5	Polycyclic	4
Imide	2	Polyether	5
Inorganic Chemical	1	Urea Compound	1

* Revised from (15) based on information presented in Appendix 1.

Table 5: Product classes tested in the ICE test method*.

Product Class	# of Chemicals	Product Class	# of Chemicals
Adhesive	2	Fertilizer	1
Antifungal	3	Food additive	1
Antihistamine	1	Fungicide / Germicide / Bactericide	8
Anti-infective	3	Industrial Chemical, Intermediate or Formulation	19
Antiseptic	2	Not Classified	23
Caustic Agent	4	Optical Resolution Agent	1

Chlorination by-product	1	Paint	4
Cleaner	8	Pesticide / Herbicide	17
Copolymer	8	Preservative	6
Cosmetic Ingredient	1	Pharmaceutical Compounds / Intermediates	6
Detergent	8	Raw Material	9
Developer	1	Reagent	4
Disinfectant	5	Resin	2
Dyes & Stains	10	Silicon Resin	1
Elastomer	2	Soap	9
Enzyme Inhibitor	1	Surfactant	25
Enzyme Solution	3	Solvent	37

* Revised from (15) based on information presented in Appendix 1.

SENSITIVITY, SPECIFICITY AND ACCURACY

20. Within the ICE validation database a total of 152 chemicals (72 substances and 80 mixtures) had sufficient *in vivo* and *in vitro* data to assess the ICE predictive capacity (Appendix 1). Their distribution according to the UN GHS classification categories are described below.

- Identification of GHS NC (Bottom-Up approach):
  152 chemicals (72 substances + 80 mixtures)
  79 NC + 73 classified chemicals (30 Cat 1 + 6 Cat 1/2 + 27 Cat 2A + 8 Cat 2B + 2 Cat 2A/2B)
- Identification of GHS Cat 1 (Top-Down approach):
  139 chemicals (65 substances + 75 mixtures)
  27 Cat 1 + 113 non-Cat 1 (26 Cat 2A + 8 Cat 2B + 79 Non-Classified (NC))

Out of the 175 chemicals from the validation dataset, a number of chemicals (n=15) had no raw *in vivo* data to allocate a UN GHS Classification. In addition, a number of chemicals (n=13) had Study Criteria Not Met (SCNM) to assign an *in vivo* classification (e.g., incomplete dataset to assess reversibility / irreversibility of effects at day 21). For a large number of them (n=11), the *in vivo* scores suggested the need for classification even if not possible to allocate a specific classification category (i.e., GHS Cat 2B versus 2A versus 1). These chemicals were used for the evaluation of the predictive capacity of the ICE test method in a bottom-up approach, but not for the top-down approach due to uncertainty as to which classification category to assign (i.e., GHS Cat 1 versus GHS Cat 2). Chemicals that had a SCNM and were estimated to be non-classified based on expert judgement (n=2), were not included in any of the analyses for precautionary reasons (although in the original evaluation they were considered as NC). A total of 5 chemicals that were classified as Eye GHS Cat 1 based on data from skin corrosion studies were not included for the purposes of the Test Guideline, in order to consider only chemicals for which high quality *in vivo* ocular data was available. Finally, two chemicals had a borderline GHS Cat 1 / Cat 2 classification so that they could only be used for the evaluation of the predictive capacity of the ICE test method in a bottom-up approach, and not in a top-down approach.

Overall Predictive Capacity

21. Due to discrepancies found in a number of *in vitro* and *in vivo* classifications from previous validation studies (for details see Appendixes 1, 2 and 3), the predictive capacities of the ICE test method were re-calculated for *i)* the identification of GHS Category 1 chemicals (Top-Down approach) and *ii)* the identification of non-classified chemicals (Bottom-up approach) as shown in Tables 6 and 7. The analyses were based on the outcome of individual test substances (and not on individual laboratory outcome), as recommended by the Expert Group on Eye Irritation, in order to be in alignment with previous ICCVAM evaluations and with the analyses carried out in the context of the revisions of the BCOP Test Guideline.

Table 6: Predictive capacity of the ICE test method for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS[1] Category 1) from all other categories.

Top-.Down Approach	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
Overall	140	86	120/140	52	14/27	48	13/27	94	106/113	6	7/113
Without alcohols, solids and surfactants	82	94	77/82	71	5/7	29	2/7	96	72/75	4	3/75

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (4): Category 1 vs. Non-Category 1 (No Category + Cat. 2B + Cat. 2A).

Table 7: Predictive capacity of the ICE test method for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS[1] Non-Classified) from all other irritant categories.

Bottom-Up Approach	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
Overall	152	82	125/152	99	72/73	1	1/73	67	53/79	33	26/79
Without anti-fouling organic-solvent containing paints	149	83	123/149	100	71/71	0	0/71	67	52/78	33	26/78

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (4): No Category vs. Classified Chemicals (Cat. 1 + Cat. 2A + Cat. 2B).

22. For the Top-Down approach, alcohols were found to risk over-prediction (4 alcohols out of 10 non-Category 1 were over-predicted as Category 1) whereas solids and surfactants were found to risk under-prediction (6 out of 11 Category 1 solids were found to be under-predicted, and 6 out of 9 Category 1 surfactants were found to be under-predicted). Table 8 shows the false positive and false negative rates

obtained for specific chemical classes and properties of interest, including mixtures and substances based on the revised dataset (Appendix 1). Substances, mixtures, liquids and solids all showed false positive rates below or equal to 15% suggesting an appropriate identification of GHS Category 1. The rate of false negatives was found to be particularly high (i.e., higher than 50% for 5 chemicals or more), for solids and surfactants. However due to the fact that the underpredicted solids and surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (as none of the GHS Cat 1 solid and surfactant materials in the validation database are underpredicted as GHS non-classified), solids and surfactants are not considered to be out of the applicability domain of the ICE test method.

23. For the Bottom-up approach, anti-fouling organic solvent containing paint were found to risk under-prediction (1 out of 2 classified anti-fouling solvent containing paint was found to be under-predicted as non-classified). As explained in paragraph 12, a warning sentence was included in the Test Guideline for this category of materials but they were not to excluded from the applicability domain of the ICE test method for the following reasons: i) there was insufficient evidence (only two classified chemicals from this category), ii) the type of exposure is unlikely to occur in humans, and iii) sticky materials present similar difficulties to test either *in vitro* and *in vivo*. Table 9 shows the false positive and false negative rates obtained for specific chemical classes and properties of interest, including mixtures and substances based on the revised dataset (Appendix 1). Substances, mixtures, liquids and solids all showed false negative rates below or equal to 5% suggesting an appropriate identification of GHS Non-classified chemicals based on the criteria discussed by the OECD Expert Group on Eye Irritation. Regarding the false positive rates, the ICE test method was found to have a lower overall false positive rate as compared to other test methods accepted for this purpose (i.e., 33% for ICE versus 69% for BCOP and 68% for CM). The false positive rates was found nevertheless to be particularly high (i.e., higher than 50% for 5 chemicals or more) for surfactants. However due to the fact that the overpredicted surfactants would need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (2) (as none of the *in vivo* GHS non-classified chemicals (n=73) was overpredicted as GHS Cat. 1), surfactants are not considered to be out of the applicability domain of the ICE test method.

Table 8: False positive and false negative rates of the ICE test method, by properties of interest, chemical class and product categories, for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS[1] Category 1) from all other categories.

Top-Down Approach	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	140	6	7/113	48	13/27
Properties of interest					
Substances	65	15	6/40	44	11/25
Mixtures	75	1	1/73	100*	2/2[4,*]
Liquids[5]	95	8	6/80	40	6/15
Solids[5]	34	0	0/23	55	6/11
Emulsions and gels[5]	7	14	1/7	n.a.	n.a.
Chemical Classes[6]					
Alcohol	12	40	4/10	50*	1/2*
Amine/Amidine	5	0*	0/1*	50*	2/4*
Carboxylic acid	10	0*	0/3*	43	3/7
Ester	9	13	1/8	0*	0/1*
Heterocyclic	9	0*	0/3*	50	3/6
Onium compound	8	0*	0/2*	50	3/6
Polyether	5	25*	1/4*	100*	1/1*

Product categories					
Cleaners	4	0*	0/3*	100*	1/1*
Copolymer	8	13	1/8	n.a.	n.a.
Detergent	7	0*	0/4*	100*	3/3*
Dyes & stains	8	0	0/7	0*	0/1*
Fungicide / Germicide / Bactericide	7	0*	0/1*	50	3/6
Industrial Chemical, Intermediate or Formulation	16	0	0/14	50*	1/2*
Pesticide / Herbicide	12	0	0/6	50	3/6
Preservative	5	0*	0/1*	25*	1/4*
Pharmaceutical compound or intermediate	4	50*	1/2*	50*	1/2*
Raw material	8	0	0/8	n.a.	n.a.
Soap	7	0	0/6	100*	1/1*
Solvent	34	19	6/31	0*	0/3*
Surfactant – Total[7] -cationic -nonionic -anionic	21 7 5 2	0 0* 0* 0*	0/12 0/1* 0/4* 0/1*	67 50 100* 100*	6/9 3/6 1/1* 1/1*

* Too small dataset to make definitive conclusions; n.a.: not applicable.
[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4] Only few formulations having severe effects are available.
[5]Physical form (*i.e.*, solid or liquid) not known for 4 chemicals.
[6]Chemical classes included in this Table are represented by at least five chemicals tested in the ICE test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh) as described in (2) and (15).
[7]Combines single substances labelled as surfactants along with surfactant-containing mixtures.

Table 9: False positive and false negative rates of the ICE test method, by properties of interest, chemical class and product categories, for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS[1] No Category) from all other irritant categories.

Bottom-Up Approach	**N[2]**	**False Positive Rate**		**False Negative Rate**	
		%	No.[3]	%	No.[3]
Overall	152	33	26/79	1	1/73
Properties of interest					
Substances	72	70	14/20	0	0/52
Mixtures	80	20	12/59	5	1/21
Liquids[4]	101	36	20/55	2	1/46
Solids[4]	38	18	3/17	0	0/21
Emulsions, gels and paste[4]	9	50*	2/4*	0	0/5
Chemical Classes[5]					
Alcohol	13	100*	4/4*	0	0/9
Amine/Amidine	6	100*	1/1*	0	0/5
Carboxylic acid	11	100*	2/2*	0	0/9

Ester	10	100*	4/4*	0	0/6
Heterocyclic	9	100*	2/2*	0	0/7
Onium compound	8	100*	1/1*	0	0/7
Polyether	5	100*	2/2*	0*	0/3*
Product categories					
Cleaners	5	100*	1/1*	0*	0/4*
Copolymer	8	33	2/6	0*	0/2*
Detergent	7	100*	2/2*	0	0/5
Dyes & stains	9	43	3/7	0*	0/2*
Fungicide / Germicide / Bactericide	8	100*	1/1*	0	0/7
Industrial Chemical, Intermediate or Formulation	18	50*	2/4*	0	0/14
Paints	4	0*	0/2*	50*	1/2*
Pesticide / Herbicide	14	50*	2/4*	0	0/10
Preservative	5	n.a.	n.a.	0	0/5
Pharmaceutical compound or intermediate	6	n.a.	n.a.	0	0/6
Raw material	9	20	1/5	0*	0/4*
Soap	8	25*	1/4*	0*	0/4*
Solvent	35	38	8/21	0	0/14
Surfactant – Total[6] -cationic -nonionic -anionic	22 7 5 2	63 100* 100* 100*	5/8 1/1* 2/2* 1/1*	0 0 0* 0*	0/14 0/6 0/3* 0/1*

* Too small dataset to make definitive conclusions; n.a.: not applicable.
[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Physical form (*i.e.*, solid or liquid) not known for 4 chemicals.
[5]Chemical classes included in this Table are represented by at least five chemicals tested in the ICE test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh) as described in (2) and (15).
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.

WITHIN- AND BETWEEN-LABORATORY REPRODUCIBILITY

24. A thorough evaluation of the ICE reproducibility was conducted in the 2003-2006 retrospective validation study (2). Based on a quantitative analysis of within-laboratory reproducibility of the ICE test method endpoints, the evaluation showed CV values for the corneal thickness measurement, when results were compared within experiments, varying from 1.8% to 6.3% (2) (3). The other endpoints evaluated produced ranges of CV values that were larger, with variability most prominent with the non-irritating substance. However, this can be explained by an exaggeration of variability given the relatively small values that were produced by chemicals not requiring classification relative to chemicals inducing eye irritation and serious eye damage (i.e., corneal swelling values of 2, 0, and 3 yield a higher CV than values of 11, 14, and 18). A similar discussion also can be applied to the variability among the qualitative endpoints (i.e., corneal opacity and fluorescein retention) given the small dynamic range of their scores (0-4 or 0-3, respectively).

25. Regarding the between-laboratory reproducibility, the retrospective studies showed median/mean % CV values to be 32%/35% for the Irritation Index, 36%/39% for fluorescein retention, 37/47% for corneal opacity, and 75%/77% for corneal swelling (2). All laboratories were in 100% agreement on the classification of 75% (44/59) of the substances according to the UN GHS classification system for both the top-down (2) and bottom-up approaches (15) according to the UN GHS classification. Finally, the EC/HO study showed the following inter-laboratory correlations between the ICE classification at TNO (lead laboratory) and the classifications obtained in three other laboratories: 0.829, 0.849 and 0.844 (11).

26. Specific issues were raised on the between-laboratory variability of the corneal swelling endpoint. This was due to the use of different slit-lamp measuring devices by the participating laboratories of the EC/HO study which, unless normalized, can contribute to the increased variability and/or the excessive values calculated for this endpoint (2). In particular, out of the four participating laboratories, two (that are no longer active in the area of toxicity testing) were reported to use different slit-lamps and different slit width settings resulting in different ranges of values for corneal swelling (see Appendix 4). In order to avoid potential variability issues linked to this endpoint, the use of a specific pachymeter and appropriate slit width, together with the use of proficiency chemicals are requested in both the adopted TG 438 (9) and the revised TG 438 (i.e., old paragraph 45, new paragraph 50: "*Corneal swelling scores are only applicable if thickness is measured with a Haag-Streit BP900 slit-lamp microscope with depth-measuring device no. I and slit-width setting at 9½, equalling 0.095 mm. Users should be aware that slit-lamp microscopes could yield different corneal thickness measurements if the slit-width setting is different.*").

Considerations on variability for the Bottom-Up approach

27. As shown in Table 7 only one chemical was identified as a false negative in the ICE test method for the identification of chemicals not requiring classification for eye irritation or serious eye damage in a Bottom-Up approach (i.e., TNO-94, a anti-fouling solvent containing paint). However, a total of eight chemicals that were correctly predicted as causing ocular effects that require a UN GHS classification, were found to be false negatives in some of the participating laboratories (Table 10).

Table 10. Chemicals showing one or more under-classification in the various participating laboratories.

N.	Chemical name	*In vivo* GHS Cat.	Physical state	Lab 22	Lab 25	Lab 24	Lab 27	Overall *in vitro* class
15	Captan 90	Cat 1	Solid	NC	2	2B	2B	2B
16	4- Carboxybenzaldehyde	Cat 2A	Solid	2B	1	NC	2	2A
36	Ethyl-2-methylacetoacetate	Cat 2B	Liquid	NC	2B	2B	NC	2B
46	Maneb	Cat 2A (EJ)	Solid	NC	2A	NC	2B	2B
50	Methyl cyanoacetate	Cat 2A	Liquid	NC	2A	NC	2B	2B
62	Quinacrine	Cat 1	Solid	2B	NC	2A	2B	2B
71	Sodium oxalate	Cat 1	Solid	2B	2B	NC	NC	2B
72	Sodium perborate	Cat 1	Solid	NC	2B	2B	2B	2B

EJ : classification based on expert judgment

Over the entire dataset, these chemicals represent 6 solids out of the 21 GHS classified solids present in the ICE validation dataset (i.e., 29%), and 2 liquids out of the 46 GHS classified liquids present in the ICE validation dataset (i.e., 4%). Due to higher probability of solids to have discordant classifications and in a

precautionary approach, the revised Test Guideline requires that "*In the case of solid materials leading to a GHS NC outcome, a second run of three eyes is recommended to confirm or discard the negative outcome*" (revised paragraph 22).

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