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STREAMLINED SUMMARY DOCUMENT SUPPORTING OECD GUIDELINE 437 ON THE BOVINE CORNEAL OPACITY AND PERMEABILITY FOR EYE IRRITATION/CORROSION

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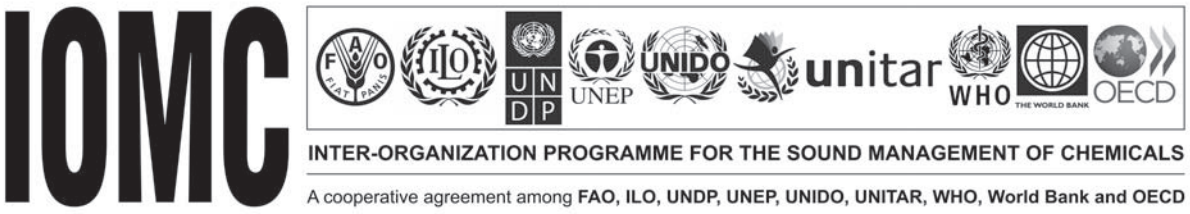

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ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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or contact:

OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This streamlined summary document (SSD) was developed to provide summary information in support of OECD Test Guideline 437 on the Bovine Corneal Opacity and Permeability 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 437 (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- and between-laboratory reproducibility of the method.

The SSD was approved by the WNT with a few changes to paragraph 12, including additional references 23, 24, 25 and 26, 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.

Description of applicability domain and performance based on the retrospective validation studies of the Bovine Corneal Opacity and Permeability (BCOP) Test Method (Test Guideline 437) 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 Bovine Corneal Opacity and Permeability (BCOP) 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 161 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 BCOP 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 in the EU at that time). On the basis of all the collected data and information the BCOP 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 (Test Guideline 405) (8), which is not (or not expected to be) fully reversible within 21 days of application. Following these recommendations, the OECD officially adopted the BCOP as OECD Test Guideline (TG) 437 for identifying chemicals inducing serious eye damage in September 2009 (9).

The 2006-2009 Validation Studies

3. Following the 2003-2006 retrospective validation study NICEATM/ICCVAM, in collaboration with ECVAM and JaCVAM, further evaluated between 2006 and 2009 the usefulness and limitations of the BCOP test method for the additional identification of chemicals (substances and mixtures) 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 in the EU at that time), *i.e.*, its usefulness and limitations for initiating a Bottom-Up approach (1).

4. The updated BCOP validation database comprises 238 studies on 213 chemicals (107 substances and 106 mixtures) (UN GHS / EU CLP: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 6 Cat 1/Cat 2, 1 No Cat/Cat 2A, 89 No Cat, 5 SCNM, 11 without *in vivo* data; US EPA: 63 Cat I, 23 Cat II, 1 Cat II/Cat III, 59 Cat III, 44 Cat IV, 13 SCNM, 11 without *in vivo* data), collected from seven individual studies (Gautheron *et al.* 1994 (10), Balls *et al.* 1995 (11), Swanson *et al.* 1995 (12), Southee 1998 (13), Swanson and Harbell 2000 (14), Bailey et al. 2004 (15), Antimicrobial Cleaning Products (AMCP) BRD (16)), which were used to determine the predictive capacity of the BCOP test method (for a comprehensive list see Annex 1). Another 54 mixtures and 13 surfactant substances reported in Casterton *et al.* 1996 (17), Gettings *et al.* 1996 (18), Swanson and Harbell 2000 (14) and in

the AMCP BRD (16) were considered by NICEATM-ICCVAM in the retrospective validation of BCOP, but were not used to calculate the predictive capacity of the test method due to incomplete *in vitro* and/or *in vivo* data (2) (3) (19). The components of each of the mixtures tested in the BCOP test method can be found in Annexes 2-5 (copied from reference 19).

5. In May 2009, NICEATM/ICCVAM convened a public meeting of an independent international scientific peer review panel (PRP) on alternative ocular safety testing methods, composed of members from several EU Member States, USA, Japan and Canada. The PRP was charged with reviewing the data compiled in view of evaluating the validation status of the BCOP test method for identifying chemicals ***not classified for eye irritation or serious eye damage*** (UN GHS No Category; EU DSD Not Classified; US EPA Category IV). The PRP concluded that the usefulness of the BCOP for the identification of chemicals not classified for eye irritation or serious eye damage depended on the intended purpose (*i.e.*, the classification system) and that the BCOP can be used for the identification of chemicals not classified for eye irritation or serious eye damage (*i.e.*, to initiate a Bottom-Up approach (1)) under the UN GHS and EU DSD classification systems for which no false negatives were identified. Since the BCOP showed 6% false negatives for the US EPA classification system, the PRP did not recommend the BCOP for this classification system (20). The final ICCVAM conclusions and recommendations on the use BCOP to identify chemicals not requiring classification for eye irritation or serious eye damage were published in a Test Method Evaluation Report (TMER), to which, amongst other documents, a revised BRD and the PRP Report were annexed (19).

BCOP Issue Paper Supporting the Adoption of TG 437 for the Identification of Chemicals Not Classified for Eye Irritation or Serious Eye Damage

6. In April 2011, following the conclusions of the 2006-2009 retrospective validation study, an OECD project for updating TG 437 (9) was included in the work plan of the OECD Test Guidelines Programme. The aim of the project was to address a possible update of TG 437 to allow its use also for the identification of chemicals not requiring classification for eye irritation or serious eye damage under UN GHS, EU CLP and EU DSD classification systems. An Issue Paper was prepared by a consultant to the OECD Secretariat with the aim to review existing BCOP data and make a recommendation on the use of TG 437 for the purpose described above (Annex 6). This document was tabled for discussion by the eye irritation/corrosion expert group at an OECD expert meeting that was held on 6-7 December 2012. The BCOP Issue Paper reviewed the BCOP data presented in the ICCVAM BCOP BRD (19) as well as other newly published data in connection with the use of BCOP to identify chemicals inducing serious eye damage as well as chemicals not requiring classification for eye irritation or serious eye damage. The BCOP Issue Paper further attempted to analyse the added value of BCOP to identify chemicals inducing serious eye damage as well as chemicals not requiring classification for eye irritation or serious eye damage, when used together with other test methods in a testing strategy. All information regarding the predictive capacity of BCOP included in this SSD is based on the data reviewed in the BCOP Issue Paper and on the analysis presented in that paper.

7. In 2013, the OECD officially adopted an updated version of TG 437 allowing the use of BCOP 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 BCOP Test Method

8. The BCOP test method (TG 437) is an organotypic *ex vivo* assay that uses living bovine corneas isolated from the eyes of freshly slaughtered animals killed for human consumption. In this test method, the irritation potential of a test substance is assessed by quantitative measurements of changes in opacity and permeability of the isolated exposed corneas, using an opacitometer and a visible light spectrophotometer, respectively (9). Both permeability and opacity are used to calculate an *in vitro* irritancy score (IVIS), which is used to assign an *in vitro* irritancy hazard classification for prediction of the *in vivo* ocular irritation potential of a test substance.

9. The prediction model used by NICEATM/ICCVAM in their evaluations of BCOP predictive capacity was based on the prediction model originally proposed by Gautheron *et al*. (1994) (10) and later modified by Balls *et al*. (1995) (11) and Southee (1998) (13). This prediction model allows for the categorisation of chemicals to four regulatory irritant categories, with cut-offs at IVIS values of 3, 25 and 55. In OECD TG 437 updated in 2013, an IIVS $\leq$ 3 is used to identify a chemical as not requiring classification for eye irritation or serious eye damage (UN GHS No Category), an IVIS > 55 is used to identify a chemical as inducing serious eye damage (UN GHS Category 1), and no prediction can be made if an 3 < IVIS $\leq$ 55 is obtained. Finally, it has also been recommended that chemicals producing permeability values equal or greater than 0.6 be identified as inducing serious eye damage (UN GHS Category 1). This may be especially important for chemicals that do not produce appreciable opacity in the isolated bovine cornea, but that can damage the epithelium and increase permeability, *e.g.*, surfactant-based personal care formulations (2) (3).

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

10. In the BCOP test method, damage to the isolated cornea is assessed by measuring corneal opacity and permeability in a short-term test that typically takes less than 8 hours to perform. The two endpoints are measured quantitatively with an opacitometer and an ultraviolet/visible (UV/VIS) spectrophotometer, respectively, after ten minutes exposure plus two hours post-exposure incubation for liquid and surfactant chemicals or after four hours of exposure to solid chemicals (2).

11. Depending on the physicochemical properties of the test substance, post-exposure measurements may be extended to 24 hours (*e.g.,* for chemicals with delayed responses). In contrast, the *in vivo* rabbit eye test involves a qualitative visual evaluation 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 BCOP, liquids, semi-solids, creams and waxes are usually applied undiluted for 10 minutes, then rinsed off the cornea, followed by a 2-hour post-exposure incubation of the cornea in assay medium. Neat surfactant substances are typically tested at a concentration of 10% w/v, while mixtures containing surfactants may be tested undiluted or diluted to an appropriate concentration depending on the relevant exposure scenario *in vivo*. The same exposure and post-exposure regime used for liquids is followed for surfactant substances and mixtures containing surfactants. Solids are usually applied as a suspension or solution (20%) for four hours, then rinsed off the cornea before opacity and permeability measurements are performed. Post-exposure incubation is not required for solids. Whether the test substance is a liquid or a solid, the entire cornea is exposed for a specified duration. In the *in vivo* rabbit eye test, liquid and solid test substances are applied to the conjunctival sac, usually in an undiluted form. Because the rabbit eye can 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 BCOP. 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. Protective mechanisms for the eye (*e.g.,* blinking, tear film) are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing. However, note that for some test substances (*e.g.,* solids), blinking can also induce mechanical damage *in vivo*, contributing to a higher degree of irritation. Thus, the BCOP test method differs from the *in vivo* rabbit eye test method in the following significant ways (2):

- The BCOP 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 in the BCOP assay, while they are assessed with qualitative observations in the *in vivo* rabbit eye test.

- Corneal exposure conditions, including test substance concentration and exposure duration, are well controlled in the BCOP assay, but subject to potentially greater variation *in vivo*,

due in part to the blink response and natural tearing of the eye in a live animal. Moreover, it should be noted that solids may lead to variable and extreme exposure conditions in the *in vivo* Draize eye irritation test, which may result in irrelevant predictions of their true irritation potential (21).

- The observation period of the BCOP assay is typically less than 24 hours, whereas ocular effects are typically evaluated in the *in vivo* rabbit eye test for a minimum of 72 hours and can extend up to 21 days.

- Reversibility/irreversibility of corneal effects induced by a test substance cannot be observed in the BCOP assay *per se*, but histological evaluation of the exposed cornea 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 (22). However, 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, are built into *in vivo* testing, but are absent in *in vitro* / *ex vivo* testing.

- The BCOP 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

12. The potential shortcomings of the BCOP test method when used to identify chemicals inducing serious eye damage (UN GHS Category 1) are based on the high false positive rates for alcohols and ketones and the high false negative rate for solids observed in the validation database (2) (3) (19). When substances within these chemical and physical classes are excluded from the database, the accuracy of the BCOP test method is substantially improved (2) (3) (19) (see Table 7 below). However, since not all alcohols and ketones are over-predicted by the BCOP test method and some are correctly predicted as UN GHS Category 1, these two organic functional groups are not considered to be out of the applicability domain of the test method. Positive results obtained with alcohols or ketones should nevertheless be interpreted cautiously due to potential over-prediction. Moreover, given the fact that some solids are also correctly predicted by the BCOP test method as UN GHS Category 1, and that all solids in the validation database that were underpredicted by BCOP showed an $3 < \text{IVIS} \leq 55$ (*i.e.*, no prediction can be made) and would therefore need to be subsequently tested with other suitable test method(s) in a sequential testing strategy (8) (Annex 6), this physical state is also not considered to be out of the applicability domain of the BCOP 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 (23). 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 (24)(25)(26). 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.

13. When used to identify chemicals not requiring classification for eye irritation or serious eye damage under the UN GHS classification system, the BCOP test method has a considerably high false positive rate (*in vivo* UN GHS No Category chemicals producing an IVIS > 3) (19). This is however not considered critical since all test substances that produce an $3 < \text{IVIS} \leq 55$ (*i.e.*, no prediction can be made) would need to be subsequently tested with other suitable test method(s) in a sequential

testing strategy. The BCOP test method shows no specific shortcomings for the testing of alcohols, ketones and solids when the purpose is to identify chemicals that do not require classification for eye irritation or serious eye damage (UN GHS No Category) (see Table 8 below).

14. Another limitation of the test method is that, although it 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 BCOP directly addresses corneal effects, which are the major driver of classification *in vivo* when considering the UN GHS classification system (Annex 6). Also, although the reversibility of corneal lesions cannot be evaluated *per se* in the BCOP 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 (22). However, further scientific knowledge is required to understand how irreversible effects not linked with initial high level injury occur. Finally, the BCOP does not allow for an assessment of the potential for systemic toxicity associated with ocular exposure.

15. The BCOP test method is not recommended for the identification of test substances that should be classified as irritating to eyes (UN GHS Category 2 or Category 2A) or test substances that should be 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 (19). For this purpose, further testing with another suitable method may be required.

Table 1: Physicochemical properties and compatibility with the BCOP

Physicochemical property	Is a material with this property compatible with the BCOP assay?
Fixative	Unknown
Solvent	Yes
Extreme pH	Yes
Gases	No
Liquids	Yes
Solid materials	Yes
Emulsions	Yes
Granular materials	No
Suspensions	Yes
Coloured materials	Yes
Diluted concentrations of chemicals	Yes
Highly viscous materials	Yes
Volatile materials	Yes
Reactive chemistries	Yes[1]
Hydrophobic/lipophilic chemicals	Yes
Neat concentrations of chemicals	Yes

[1]Reactive chemistries may require the use of extended post-treatment incubations, up to 24 hours, prior to the opacity and permeability endpoints for expression of effects. Histopathology is highly recommended for such studies.

Applicability Domain

16. The Test Guideline can be used for testing all types of substances and mixtures, including those outside the chemical classes defined by the chemicals used in the validation study, provided there is no evidence that the method is not valid for the chemical tested.

Categories of Irritancy

17. Based on the conclusions of the 2003-2006 and 2006-2009 retrospective validation studies (3) (19), TG 437 was approved for classification of chemicals inducing serious eye damage (UN GHS Category 1) (in 2009), as well as 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, in 2013.

Potential Role in an ITS

18. The BCOP can be used as a validated *ex vivo* test method in a tiered testing approach as recommended in the supplement 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) (Annex 6). Due to its low specificity when used to identify chemicals not requiring classification for eye irritation or serious eye damage, the BCOP should not be the first choice to initiate a Bottom-Up approach (1), if other validated and accepted *in vitro* methods with similar high sensitivity but higher specificity are available.

19. Two studies have looked at the combination of BCOP with other *in vitro* test methods in testing strategies for the prediction of ocular hazards. Kolle et al. (2011) (27) combined BCOP with EpiOcular™ EIT (the latter can only be used to identify UN GHS No Category) in a two-tier Bottom-up/Top-Down test strategy and Hayashi et al. (2012) (28) combined BCOP with EpiOcular™ EIT, the Short Time Exposure (STE)[1] test method and the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM)[2] in a two-stage Bottom-Up tiered approach. In the data published by Kolle et al. (2011), it can be seen that chemical 10/0229-1, a UN GHS No Category ("non-irritant") solid organophosphate, is overpredicted by EpiOcular™ EIT but correctly predicted by BCOP. By using BCOP to identify both UN GHS Category 1 and UN GHS No Category chemicals, this chemical can be correctly identified as "non-irritant" using either a Bottom-Up testing strategy (EpiOcular™ EIT first followed by BCOP) or a Top-Down testing strategy (BCOP first followed by EpiOcular™ EIT) (Annex 6) (27). Hayashi et al. tested 26 UN GHS No Category chemicals from the BCOP validation database in EpiOcular™ EIT, STE and HET-CAM. Of these, 13 were overpredicted as irritant by EpiOcular™ EIT (50% false positives). Two of the 13 EpiOcular™ EIT false positives (EDTA di-potassium salt, CAS # 25102-12-9; triethanolamine, CAS # 102-71-6) are correctly identified by BCOP as not requiring hazard classification and labelling (UN GHS No Category). HET-CAM also correctly identifies two other

[1]The STE has undergone a full validation study in Japan, coordinated by JaCVAM, and an international peer-review of the study is currently being organised by ICCVAM. The STE was also submitted by Japan to the OECD for the development of a new Test Guideline, and was included in the OECD work plan for the Test Guidelines Programme in 2011.

[2]The HET-CAM was evaluated by ICCVAM together with BCOP and ICE in the retrospective validation studies of 2003-2006 (3) and 2006-2009 (19). ICCVAM considered that the HET-CAM might be useful for identifying chemicals not requiring hazard classification and labeling for eye irritation but did not recommend the test method due to lack of data in the mild/moderate irritancy range. ICCVAM recommended that additional data be collected on mild and moderate irritants to more adequately characterise the usefulness of HET-CAM. Following this recommendation, an international workshop on HET-CAM was recently held at the BfR in Berlin to help advance regulatory acceptance of the test method.

EpiOcular™ EIT false positives as "non-irritant" (1-nitropropane, CAS # 108-03-2; 1-phenyl-3-pyrazolidinone, CAS # 92-43-3), but in this case at the expense of 1 UN GHS Category 2A chemical being underclassified as No Category (4-carboxybenzaldehyde, CAS # 619-66-9). However, if a Top-Down approach would be used to test this chemical, starting with BCOP, it would be overclassified as UN GHS Category 1 instead of underclassified as UN GHS No Category. Finally, STE correctly identified 8 of 10 EpiOcular™ EIT false positives tested as "non-irritant" (including both chemicals correctly identified by BCOP but not those identified by HET-CAM), but at the same it underpredicted 3 UN GHS Category 2A and 1 Category 2B as "non-irritants". If a Top-Down approach would be used to test these four chemicals, starting with BCOP, two of them (ethanol, CAS # 64-17-5, and isopropanol, CAS # 67-63-0) would be overclassified as UN GHS Category 1 but the other two (methyl acetate, CAS # 79-20-9; ammonium nitrate, 6484-52-2) would still be underclassified as UN GHS No Category since for these, BCOP produced an IVIS between 3 and 55 (*i.e.*, no prediction can be made) (24). Methyl acetate produced a mean IVIS of 54.9 in the EC-HO validation study, which is borderline for the cut-off of 55 used to identify UN GHS Category 1. In fact, the same chemical was predicted as UN GHS Cat 1 by BCOP at BASF with an IVIS of 58.1 (27). By using BCOP to identify both UN GHS Category 1 and UN GHS No Category chemicals in combination with EpiOcular™ EIT, the false positive rate for chemicals not requiring classification for eye irritation or serious eye damage would be reduced by 7.7%, from 13/26 (50%) to 11/26 (42.3%). If HET-CAM is further integrated in the strategy, the false positive rate would be further reduced to 34.6% (9/26). If STE would also be integrated in the test strategy despite its higher false negative rate, the false positive rate would decrease to only 11.5% (3/26) (Annex 6) (28).

Mode of Action (MoA)

20. 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.*, alkylation, oxidative attack on macromolecules such as essential proteins or nucleic acids). The BCOP 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 2: Summary of events involved in chemical-induced eye irritation *in vivo* (text in italics represents irreversible responses) with information on whether they are modelled by the BCOP test method or not.

Events involved in chemical-induced eye irritation	Modelled by the BCOP 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	Unknown
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 (corneal epithelium); No (limbus)
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.	No[1]
Recovery from response, *i.e.*, length of time for cell responses to return to control levels	No

[1]In OECD TG 437, a time course is not specified. However, a time course for exposures, as well as a time course for post-exposure expression of changes, up to 24 hours, can be designed into the assay.

Chemical Classes

21. A total of 213 individual chemicals (107 substances and 106 mixtures) were evaluated in the 2006-2009 test method evaluation (19), including representatives from a number of chemical and product classes that have been evaluated using *in vivo* rabbit eye data. Chemicals with a wide range of individual responses have been evaluated. Test data were collected from Gautheron *et al.* (1994) (10), Balls *et al.* (1995) (11), Swanson *et al.* (1995) (12), Southee (1998) (13), Swanson and Harbell (2000) (14), Bailey *et al.* (2004) (15) and the AMCP BRD (16) (Annex 1). The ICCVAM PRP (20) concluded that "*the chemical database appeared adequate, however, additional chemicals in certain chemical classes will provide a more robust statistical inference as these data become available*", however, the PRP did not specifically identify any chemicals or product classes as being of special concern. For a complete list of chemical and product classes see Annex 1. The components of each of the mixtures tested in the BCOP test method can be found in Annexes 2-5 (copied from reference 19).

22. Tables 3 and 4 show the chemical and product classes for the chemicals included in the BCOP validation database. Information, including chemical name, Chemical Abstracts Service

Registry Number (CASRN), chemical and/or product class, concentration(s) tested, purity, supplier or source, and literature reference using the chemical are provided in Annex 1.

Table 3: Chemical classes tested in the BCOP test method.

Chemical Class	# of Chemicals	Chemical Class	# of Chemicals
Acyl halide	3	Imide	2
Alcohol	22	Inorganic salt	6
Aldehyde	1	Ketone	12
Alkali	3	Lactone	3
Aluminum compound	1	Nitrile compound	1
Amide	2	Nitro compound	2
Amidine	6	Oil	1
Amine	10	Onium compound	12
Amino acid	4	Organic salt	3
Boron compound	1	Organic sulfur compound	5
Carboxylic acid	17	Organophosphate	1
Ester	12	Organosilicon compound	1
Ether/Polyether	9	Phenol	1
Formulation	69	Polycyclic compound	3
Heterocyclic compound	12	Terpene	1
Hydrocarbon	18	Wax	1

Table 4: Product classes tested in the BCOP test method.

Product Class	# of Chemicals	Product Class	# of Chemicals
Adhesive	1	Fertilizer	1
Agricultural chemical	2	Flame retardant	1
Antifreeze agent	1	Flavor ingredient	3
Antimicrobial cleaning product	66	Food additive	1
Bactericide/Fungicide/ Disinfectant/Germicide	11	Herbicide	3
Beverage	1	Insect repellant	8
Bleach	3	Lubricant/lubricant additive	6
Chelating agent	2	Paint, lacquer, varnish (component)	1
Chemical/synthetic intermediate	28	Pesticide	8
Cleaner	15	Petroleum product	16
Cleanser (personal care)	13	Photographic chemical/ developing agent	2
Coupling agent	1	Plant growth regulator	2
Cutting fluid	2	Plasticizer	4
Degreaser	1	Preservative	2
Dessicant	1	Reagent	5
Detergent	11	Shampoo (hair)	14
Drug/Pharmaceutical/ Therapeutic agent and/or metabolite	17	Soap	3

Dry cleaning preparation	1	Solvent	34
Dye, in manufacture of	3	Surfactant	39
Emulsifier	1	Anionic surfactant	3
Etching and/or electroplating	2	Cationic surfactant	6
Explosive	1	Nonionic surfactant	5
Fabric softener	1	Thermometer fluid	1

Sensitivity, Specificity and Accuracy:

23. The updated BCOP validation database contains a total of 213 chemical, most of which had sufficient *in vivo* data to be assigned an ocular irritancy classification according to the UN GHS classification system (4), sufficient for the calculation of accuracy to identify UN GHS Category 1 (Top-Down) (191 chemicals: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 1 No Cat/Cat 2A and 89 No Cat) or UN GHS No Category (Bottom-Up) (196 chemicals: 65 Cat 1, 32 Cat 2A, 3 Cat 2B, 1 Cat 2, 6 Cat 1/Cat 2 and 89 No Cat). The difference in the number of chemicals used in the two calculations (191 vs. 196) derives from (i) the use of 1 chemical (Tetraaminopyrimidine sulphate) that has two *in vivo* studies, one indicating UN GHS No Category (ECETOC) and the other UN GHS Category 2A (Gautheron et al. 1996 (10)), for the Top-Down calculations but not for the Bottom-Up calculations; and (ii) the use of 6 chemicals with *in vivo* Study Criteria Not Met (SCNM), but for which one can be certain they are either UN GHS Category 2 or UN GHS Category 1, for the Bottom-Up calculations but not for the Top-Down calculations (see Annex 1). In order to calculate the appropriate ocular irritancy hazard classification, detailed *in vivo* data consisting of cornea, iris, and conjunctiva scores for each animal at 24, 48, and 72 hours following test substance administration and/or assessment of the presence or absence of lesions at 7, 14, and 21 days were evaluated. Some of the test substances had insufficient *in vivo* data to assign a hazard classification (16 chemicals: 5 with study criteria not met, 11 without *in vivo* data). These chemicals were therefore not used to evaluate test method accuracy.

Table 5: False positive and false negative rates of the BCOP test method, by chemical class and properties of interest, for the UN GHS[1] classification system for distinguishing chemicals (substances and mixtures) inducing serious eye damage (UN GHS Category 1) from all other categories.

Category	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	191	25	32/126	14	9/65
Chemical Class[4]					
Alcohol	17	50	7/14	67	2/3
Amine/Amidine	7	0	0/4	0	0/3
Carboxylic acid	14	37	3/8	33	2/6
Ester	10	12	1/8	0	0/2
Ether/Polyether	6	40	2/5	0	0/1
Heterocycle	13	14	1/7	17	1/6
Hydrocarbon	11	9	1/11	-	0/0
Inorganic salt	7	0	0/3	0	0/4
Ketone	9	44	4/9	-	0/0
Onium compound	11	0	0/3	12	1/8
Properties of Interest					
Liquids[5]	150	26	27/102	8	4/48
Solids[5]	33	15	3/20	38	5/13
Pesticide	8	66	2/3	40	2/5
Surfactant – Total[6]	75	17	6/35	7	3/40
-nonionic	25	21	3/14	9	1/11
-anionic	22	0	0/12	10	1/10
-cationic	7	0	0/1	0	0/6
pH – Total[7]	34	21	4/19	13	2/15
- acidic (pH < 7.0)	13	27	3/11	0	0/2
- basic (pH > 7.0)	16	0	0/3	15	2/13
- equals 7	3	33	1/3	-	0/0
Category 1 Subgroup[8] - Total	52[9]	-	-	17	9/52
- 4 (CO=4 at any time)	25	-	-	16	4/25
- 3 (severity/persistence)	1	-	-	0	0/1
- 2 (severity)	3	-	-	33	1/3
- 2-4 combined[10]	29	-	-	17	5/29
- 1 (persistence)	23	-	-	17	4/23

[1]GHS = Globally Harmonized System (UN 2011) (4).
[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Chemical classes included in this table are represented by at least five substances tested in the BCOP test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh).
[5]Physical form (*i.e.*, solid or liquid) not known for some chemicals, and therefore the overall number does not equal the sum of the solid and liquid chemicals.
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.
[7]Total number of chemicals for which pH information was obtained.
[8]NICEATM-defined subgroups assigned based on the lesions that drove classification of a UN GHS Category 1 chemical. 1: based on lesions that are persistent; 2: based on lesions that are severe (not

including Corneal Opacity [CO]=4); 3: based on lesions that are severe (not including CO=4) and persistent; 4: CO = 4 at any time.

[9]The number of chemicals evaluated in the Category 1 subgroup analysis may be less than the total number of *in vivo* Category 1 chemicals evaluated, since some chemicals could not be classified into the subgroups used in the evaluation.

[10]Subcategories 2 to 4 combined to allow for a direct comparison of UN GHS Category 1 chemicals classified *in vivo* based on some lesion severity component and those classified based on persistent lesions alone.

Table 6: False positive and false negative rates of the BCOP test method, by chemical class and properties of interest, for the UN GHS[1] classification system for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage (UN GHS No Category) from all other irritant categories.

Category	N[2]	False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]
Overall	196	69	61/89	0	0/107
Chemical Class[4]					
Alcohol	17	0	3/7	0	0/10
Amine/Amidine	7	25	1/4	0	0/3
Carboxylic acid	14	83	5/6	0	0/8
Ester	10	75	3/4	0	0/6
Ether/Polyether	6	25	1/4	0	0/2
Heterocycle	13	67	4/6	0	0/7
Hydrocarbon	11	73	8/11	-	0/0
Inorganic salt	7	50	1/2	0	0/5
Ketone	9	67	4/6	0	0/3
Onium compound	11	100	2/2	0	0/9
Properties of Interest					
Liquids[5]	154	75	54/72	0	0/82
Solids[5]	34	38	6/16	0	0/18
Pesticide	8	100	2/2	0	0/6
Surfactant – Total[6]	75	80	24/30	0	0/45
-nonionic	25	69	9/13	0	0/12
-anionic	23	82	9/11	0	0/12
-cationic	7	100	1/1	0	0/6
pH – Total[7]	34	78	7/9	0	0/25
- acidic (pH < 7.0)	13	75	3/4	0	0/9
- basic (pH > 7.0)	16	100	3/3	0	0/13
- equals 7	3	-	0/0	0	0/3

[1]GHS = Globally Harmonized System (UN 2011) (4).

[2]N = Number of chemicals.
[3]Data used to calculate the percentage.
[4]Chemical classes included in this table are represented by at least five substances tested in the BCOP test method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh).
[5]Physical form (*i.e.*, solid or liquid) not known for some chemicals, and therefore the overall number does not equal the sum of the solid and liquid chemicals.
[6]Combines single substances labelled as surfactants along with surfactant-containing mixtures.
[7]Total number of chemicals for which pH information was obtained.

Overall Predictive Capacity

Table 7: Predictive capacity of the BCOP test method for distinguishing chemicals (substances and mixtures) inducing serious eye damage from all other categories, as defined by the UN GHS classification system[1] (based on Table 2 in the BCOP Issue Paper; Annex 6).

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	191	79	150/191	86	56/65	14	9/65	75	94/126	25	32/126
Substances	100	73	73/100	77	20/26	23	6/26	72	53/74	28	21/74
Mixtures	91	85	77/91	92	36/39	8	3/39	79	41/52	21	11/52
All without alcohols, ketones and solids	131	85	111/131	92	46/50	8	4/50	80	65/81	20	16/81
Substances without alcohols, ketones and solids	42	83	35/42	83	10/12	17	2/12	83	25/30	17	5/30
Mixtures without alcohols, ketones and solids	89	85	76/89	95	36/38	5	2/38	78	40/51	22	11/51

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): Category 1 vs. No Category/Category 2A/2B.

Table 8: Predictive capacity of the BCOP test method for distinguishing chemicals (substances and mixtures) not requiring classification for eye irritation or serious eye damage from all other irritant categories, as defined by the UN GHS classification system[1] (based on Table 3 in the BCOP Issue Paper; Annex 6).

Chemicals	No.	Accuracy		Sensitivity		False Negatives		Specificity		False Positives	
		%	No.	%	No.	%	No.	%	No.	%	No.
All	196	69	135/196	100	107/107	0	0/107	31	28/89	69	61/89
Substances	104	72	75/104	100	55/55	0	0/55	41	20/49	59	29/49
Mixtures	92	65	60/92	100	52/52	0	0/52	20	8/40	80	32/40
All without alcohols, ketones and solids	135	64	87/135	100	75/75	0	0/75	20	12/60	80	48/60
Substances without alcohols, ketones and solids	45	64	29/45	100	24/24	0	0/24	24	5/21	76	16/21
Mixtures without alcohols, ketones and solids	90	64	58/90	100	51/51	0	0/51	18	7/39	82	32/39

Abbreviations: No. = data used to calculate the percentage.
[1]UN GHS classification system (UN 2011) (4): No Category vs. Category 1/2A/2B.

Within- and Between-Laboratory Reproducibility:

24. A thorough evaluation of the BCOP reliability was conducted in the 2003-2006 retrospective validation study concerning the use of BCOP for identifying chemicals inducing serious eye damage (2) (3). This evaluation showed a median coefficient of variation (CV) for IVIS for replicate corneas (n=3) within individual experiments ranging from 11.8% to 14.2% in one study, and mean and median CV values for IVIS for replicate corneas (n=4) within individual experiments of 71% and 35%, respectively, in a second study. The between experiment mean CV values of IVIS for 16 chemicals tested two or more times in three laboratories ranged from 12.6% to 14.8%, while the median CV values ranged from 6.7% to 12.4%.

25. Additional analyses of between-laboratory reproducibility were also conducted in the second retrospective validation study (2006-2009) to evaluate the extent of agreement of BCOP hazard

classifications among the laboratories that participated in three different inter-laboratory validation studies (10) (11) (13) (19). These analyses showed nearly 100% agreement of classification among the different laboratories in each study, confirming the results that had been obtained in the first evaluation. The extent of agreement was 100% when distinguishing chemicals inducing serious eye damage from all other ocular hazard categories for 72% (91/127) of the chemicals according to the UN GHS classification system. When distinguishing chemicals not requiring classification for eye irritation or serious eye damage from all other ocular hazard categories, the extent of agreement was 100% for 80% (103/128) of the chemicals according to the UN GHS classification system (19).

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