The EpiOcular™ Eye Irritation Test is the Method of Choice for the *In Vitro* Eye Irritation Testing of Agrochemical Formulations: Correlation Analysis of EpiOcular Eye Irritation Test and BCOP Test Data According to the UN GHS, US EPA and Brazil ANVISA Classification Schemes

Susanne N. Kolle,[1] Maria Cecilia Rey Moreno,[1] Winfried Mayer,[2] Andrew van Cott,[3] Bennard van Ravenzwaay[1] and Robert Landsiedel[1]

[1]BASF SE Experimental Toxicology and Ecology, Ludwigshafen, Germany; [2]BASF SE Agricultural Products Formulation Development, Ludwigshafen, Germany; [3]BASF Corporation, Research Triangle Park, USA

Summary — The Bovine Corneal Opacity and Permeability (BCOP) test is commonly used for the identification of severe ocular irritants (GHS Category 1), but it is not recommended for the identification of ocular irritants (GHS Category 2). The incorporation of human reconstructed tissue model-based tests into a tiered test strategy to identify ocular non-irritants and replace the Draize rabbit eye irritation test has been suggested (OECD TG 405). The value of the EpiOcular™ Eye Irritation Test (EIT) for the prediction of ocular non-irritants (GHS No Category) has been demonstrated, and an OECD Test Guideline (TG) was drafted in 2014. The purpose of this study was to evaluate whether the BCOP test, in conjunction with corneal histopathology (as suggested for the evaluation of the depth of the injury) and/or the EpiOcular-EIT, could be used to predict the eye irritation potential of agrochemical formulations according to the UN GHS, US EPA and Brazil ANVISA classification schemes. We have assessed opacity, permeability and histopathology in the BCOP assay, and relative tissue viability in the EpiOcular-EIT for 97 agrochemical formulations with available *in vivo* eye irritation data. By using the OECD TG 437 protocol for liquids, the BCOP test did not result in sufficient correct predictions of severe ocular irritants for any of the three classification schemes. The lack of sensitivity could be improved somewhat by the inclusion of corneal histopathology, but the relative viability in the EpiOcular-EIT clearly outperformed the BCOP test for all three classification schemes. The predictive capacity of the EpiOcular-EIT for ocular non-irritants (UN GHS No Category) in the 97 agrochemical formulations tested (91% sensitivity, 72% specificity and 82% accuracy for UN GHS classification) was comparable to that obtained in the formal validation exercise underlying the OECD draft TG. We therefore conclude that the EpiOcular-EIT is currently the best *in vitro* method for the prediction of the eye irritation potential of liquid agrochemical formulations.

Key words: *agrochemical formulations, BCOP, Draize test, EIT, EpiOcular, eye irritation,* in vitro *testing.*

Address for correspondence: *Susanne N. Kolle, BASF SE, Experimental Toxicology and Ecology, Carl Bosch Straße 38, 67056 Ludwigshafen, Germany.*
E-mail: *susanne.kolle@basf.com*

Introduction

Eye irritation is one of the acute toxicity endpoints that needs to be addressed when registering agrochemical formulations. To date, the Draize rabbit eye test (1) is the only test that is accepted worldwide by regulators for the determination of the full range of eye irritation potential. Performed on a whole organism, this animal test has been increasingly criticised with regard to the subjectivity of the endpoints, high inter-experimental variability, questionable inter-species transferability, and animal welfare concerns (2). Meanwhile, some *in vitro* alternative methods have gained (or are close to gaining) regulatory acceptance for the identification of severe ocular irritants and ocular non-irritants, including the Bovine Corneal Opacity and Permeability (BCOP) test (3–5), which has been adopted for the testing of substances and mixtures. In this context, a 'mixture' is understood to be a mixture or a solution composed of two or more substances, in which the substances do not react (6). Hence agrochemical formulations can generally be regarded as within the applicability domain of these tests. Agrochemical formulations can typically be distinguished as being liquid (solvent-based or water-based) or solid formulations. The biological activity of such a formulation is determined by its active ingredient, which is usually formulated with other (biologically inert) materials in order to facilitate its application in the field, or its wetting or penetration of the plants or target organisms. Other components can include solvents, mineral clays, stickers, wetting agents, dispersing agents, anti-foam agents, bactericides or

other adjuvants. Formulation improves the properties of an active ingredient for handling, storage and application purposes, and may substantially influence effectiveness and safety of the final product.

No systematic analysis of the usefulness of the BCOP test and the EpiOcular™ Eye Irritation Test (EIT) methods to assess agrochemical formulations has been reported, other than data on 11 agrochemical formulations included in the in-house validation exercises of the BCOP test and EpiOcular-EIT (7, 8). Other *in vitro* tests that have been described for the assessment of the eye irritation potential of agrochemicals and, specifically, agrochemical formulations, include the Isolated Chicken Eye (ICE; 9, 10) and the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM; 11) tests. While the data on agrochemical formulations tested in the ICE are quite limited (OECD TG 438; 4), 50 agrochemical formulations were included in the study by Schrage *et al.* (11), which used the HET-CAM assay to identify ocular corrosives (GHS Category 1). The overall accuracy of this data set was 42%, with 40% false negatives and 70% false positives, indicating that the HET-CAM assay should not be used as the method of choice to identify ocular corrosive (GHS Category 1) agrochemical formulations.

The Draize rabbit eye test is the only method with full worldwide regulatory acceptance, hence it is used as a reference for the validation of *in vitro* methods. The test is often criticised for its high variability, somewhat subjective scoring, and poor predictions of human ocular irritation. For regulatory purposes, the results of the Draize test are translated into different irritation classes, but this translation is partly inconsistent among the different classification schemes, e.g. those used by the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS), the United States Environmental Protection Agency (US EPA), and the Brazilian Health Surveillance Agency, Agência Nacional de Vigilância Sanitária (Brazil ANVISA). In principle, the Draize rabbit eye test (1) distinguishes between reversible and irreversible ocular lesions, and provides a scoring system for the relative categorisation of the severity of reversible effects. At 1, 24, 48 and 72 hours, and 7, 14 and 21 days after test substance application, the reaction of the conjunctiva (redness and chemosis), cornea (opacity and area involved) and iris are scored. Depending on the legal framework, there are differences in the classification of irritant responses evaluated by various regulatory agencies (see Table 1):

— According to the UN GHS scheme, a single harmonised hazard category (Category 1) is assigned to substances that cause severe eye irritation. Substances that induce reversible eye irritation are assigned to Category 2, with the option of sub-categorisation, depending on the time required for reversal of the irritant effects (12).

— US EPA Category I substances are defined as corrosive or severe irritants (equivalent to UN GHS Category 1), while classification from II to IV is based on decreasing irritation severity, as

Table 1: Overview of UN GHS, US EPA and ANVISA classification criteria for eye irritation

	Not classified	**Category 2B**	**Category 2A**	**Category 1**
UN GHS	Minimal effects	Corneal opacity ≥ 1 and/or iritis ≥ 1 and/or conjunctival redness ≥ 2 and/or chemosis ≥ 2[a] reversible within **7 days**	Corneal opacity ≥ 1 and/or iritis ≥ 1 and/or conjunctival redness ≥ 2 and/or chemosis ≥ 2[a] reversible within **21 days**	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting (corneal opacity ≥ 3.0 and/or iritis ≥1.5)[a] for more than **21 days**
	Category IV	**Category III**	**Category II**	**Category I**
US EPA	Minimal effects clearing in **less than 24 hours**	Corneal opacity ≥ 1 and/or iritis ≥ 1 and/or conjunctival redness ≥ 2 and/or chemosis ≥ 2[b] clearing in **7 days or less**	Corneal opacity ≥ 1 and/or iritis ≥ 1 and/or conjunctival redness ≥ 2 and/or chemosis ≥ 2[b] clearing in **8–21 days**	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than **21 days**
	Toxicity class IV	**Toxicity class III**	**Toxicity class II**	**Toxicity class I**
Brazil ANVISA	No corneal opacity and irritation reversible within **24 hours**	No corneal opacity, irritation reversible within **72 hours**	No corneal opacity, irritation reversible within **7 days**	Corneal opacity (reversible or not) or persistent irritation

[a]*Calculated as the mean score following the gradings at 24, 48, and 72 hours after instillation of the test material in at least two of three tested animals.*
[b]*Maximum score in any animal.*

well as the time required for any irritation to clear. Irritation that clears in 8–21 days is classified as Category II, while irritation that clears within seven days is classified as Category III. For Category IV substances, irritation clears within 24 hours (13).

— In Brazil, agrochemical formulations are classified according to *Portaria Nº 03-MS-SNVS, de 16 de Janeiro de 1992* (*Annex III*; referred to as the Brazil ANVISA classification in this paper; 14). Agrochemical formulations are assigned a Toxicity Class I (extremely toxic) in the presence of corneal opacity, irrespective of the persistence or reversibility of the corneal effect. Toxicity Classes II to IV are assigned to test substances only in the absence of any corneal opacity, and depend on the time needed for the reversibility of the irritation.

Here, we describe the evaluation of the BCOP test in conjunction with histopathology, and the EpiOcular-EIT, in terms of their prediction of the ocular irritation potential of 97 agrochemical formulations according to different classification schemes, and for which *in vivo* data had been previously generated for regulatory purposes.

Materials and Methods

Test substances

In this study, 97 liquid agrochemical formulations were used. For all the tests, identical batches or batches with identical composition were used. An international coding system is available for the more than 60 different types of formulations (15): the nine liquid formulation types used in this study comprised capsule suspension (CS), emulsifiable concentrate (EC), flowable concentrate for seed treatment (FS), oil dispersion (OD), suspension concentrate (= flowable concentrate; SC), suspo-emulsion (SE), soluble concentrate (SL), technical concentrate (TK), and mixed formulations of CS and SC (ZC) type formulations.

Draize rabbit eye irritation test

Eye irritation data for the agrochemical formulations were obtained for regulatory purposes over approximately the past 10 years by using the Draize rabbit eye irritation test (16) and modifications thereof (1). The Draize test evaluates the ocular reaction to a test material by grading the lesions in the conjunctiva (redness and chemosis), cornea (opacity and area involved), and iris. By using Draize rabbit eye test data, the full range of eye irritation potential including irreversible (i.e. corrosion/severe irritation) and reversible effects (mild, moderate irritation) was determined. All the *in vivo* tests were performed according to the Organisation for Economic Co-operation and Development Test Guideline (OECD TG) 405 (1), and with the consent and provisions of the German animal welfare regulations in an AAALAC-certified laboratory of BASF SE. No *in vivo* experiments were performed for the purpose of this paper.

Bovine Corneal Opacity and Permeability (BCOP) assay

The BCOP test consists of an organotypic model that involves the use of bovine eyes from slaughtered cattle (17). The test has gained regulatory acceptance for the identification of severe ocular irritants (UN GHS Category 1) and ocular non-irritants (not requiring classification according to UN GHS, i.e. UN GHS No Category) of single-component test substances and multi-component mixtures (3). Since severe ocular irritation needs to be avoided for agrochemical formulations, the goal of this study with regard to the BCOP test was to evaluate whether the BCOP test in conjunction with corneal histopathology is useful to predict ocular irritation of the most severe classification (i.e. UN GHS Category 1, EPA Class I and Brazil ANVISA Toxicity Class I). In the standard BCOP test, corneal opacity is experimentally determined by the amount of light transmitted through the bovine cornea. As a second endpoint, permeability is determined by the amount of fluorescein dye that passes through the cornea. According to the current OECD TG, opacity and permeability are used to calculate the *in vitro* irritation score (IVIS = opacity + [15 × permeability]; 3). According to OECD TG 437 and the GHS classification rules, substances with an IVIS > 55 are to be regarded as severe irritants (UN GHS Category 1); substances with an IVIS ≤ 3 are to be considered ocular non-irritants; and for substances resulting in 3 < IVIS ≤ 55, no prediction can be made. Although not part of the regulatory-accepted test protocol, corneal histopathology has been suggested as a useful additional endpoint in the BCOP assay (3, 18). The corneal histopathology has been established in the Toxicology Department of BASF SE, and validated in-house to test 60 test substances for severe ocular irritation. Details of the protocols are described in Kolle *et al.* (7) and Schrage *et al.* (8). Briefly, each test substance, or control, was applied to triplicate corneas (n = 3) unless otherwise stated. The OECD TG exposure protocol for liquids was followed, i.e. corneas were exposed to 750µl of the undiluted agrochemical formulations for 10 minutes. Resulting opacity, permeability and IVIS values were summarised as arithmetic means and standard deviations.

Histopathology

The histopathological evaluation described here is based on the model of depth of injury, supported by Jester *et al.* (19, 20) and Maurer *et al.* (21). They demonstrated that the extent and depth of the initial corneal injury *in vivo* is predictive of the degree and duration of the lesion, and proposed the use of this hypothesis in *ex vivo* or *in vitro* systems to predict corneal irritation. Directly after determination of opacity and permeability, the corneas (triplicates of control and test substance-treated) were fixed in 10% neutral buffered formalin for at least 24 hours. After fixation, two to three parallel tissue sections (3–4mm wide) along the whole diameter of the cornea were cut. Standard histotechnical processing for light microscopy was performed: dehydration in alcohol solutions, clearing in xylol, embedding in paraffin, sectioning (3µm-thick) and staining with Haematoxylin–Eosin. To assess the severity of the corneal injury, an ordinal semi-quantitative grading system called the Histopathological Score of Irritation (HSI) was created (Rey Moreno *et al.*, manuscript in preparation). Different grades of severity, ranging from 0 to IV, were determined based on the depth of the injury. Corneas with a histopathological score of IV, (deepest type of injury) were considered to have severe irritation (full epithelial thickness affected, and/or swelling of the stroma > 50% of depth, and/or keratocyte changes). Corneas with HSI I, II and III were considered to overall have non-severe irritation (epithelial damage ranging from the squamous to the wing cell layer without affecting the basal layer, and/or swelling of the stroma up to 50% of depth). Corneas without damage after exposure were considered to have no irritation (HSI 0). The results of the histopathological evaluation were summarised as medians of the three corneas evaluated.

EpiOcular™ Eye Irritation Test (EIT)

The EpiOcular-EIT has undergone a formal validation exercise led by the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), and a draft OECD TG became available in July 2014 (5). The EpiOcular-EIT was established in the Toxicology Department of BASF SE, and validated in-house, in combination with the BCOP assay, to test for ocular non-irritants using 60 test substances (7). EpiOcular™ is a commercially available 3-D reconstructed human corneal model (OCL-200; purchased from MatTek Corporation, Ashland, MA, USA or MatTek In Vitro Life Science Laboratories, Bratislava, Slovak Republic). In the EpiOcular-EIT, decreasing cellular viability is used as marker for increasing eye irritating potency. In the prediction models described, a test substance that reduces the mean tissue viability to ≤ 60% after a single time point exposure is classified as irritant (GHS Category 1, 2A, or 2B, denoted as GHS Category 1/2 in this paper); if the mean tissue viability is > 60%, the test substance is predicted to be non-irritant (GHS No Category; 5, 22). Details of the protocols are described in Kolle *et al.* (7) and Kaluzhny *et al.* (22). Briefly, each test substance, or control, was topically applied to duplicate tissues (n = 2) by using the protocol for liquids (50µl of undiluted agrochemical formulation for 90 minutes). Relative viabilities were determined in relation to the negative control. The results were summarised as means and inter-tissue differences.

Statistical analysis

Data were summarised in an Excel spreadsheet, including the classification according to the US EPA, UN GHS, Brazil ANVISA schemes (based on the *in vivo* Draize rabbit eye irritation test), and *in vitro* test results (mean BCOP opacity, mean BCOP permeability, median BCOP HSI, mean relative viability obtained in the EpiOcular-EIT) as variables in columns (see Table 2). For the BCOP endpoints, three individual corneas were treated with the same test substance, or control; in the EpiOcular-EIT the relative viability values obtained resulted from two replicates per test substance or control. Replicates were processed by calculating the arithmetic mean (opacity, permeability and relative viability) or median (histopathology). All calculations were performed with Microsoft Office Excel® 2010. After assessment of the classification for eye irritating potential according to the criteria described above, the numbers of agrochemical formulations correctly or incorrectly identified by the *in vitro* methods were determined for the different classification schemes. The numbers of correctly identified positives (CP), correctly identified negatives (CN), false positives (FP) and false negatives (FN) were determined, and, by using these values, the overall accuracy or concordance (= [CP + CN] / all × 100%), the percentage of false negatives (FNR = FN / [CP + FN] × 100%) and the percentage of false positives (FPR = FP / [CN + FP] × 100%) were calculated. Calculations for the sensitivity (= 100% – FNR) and specificity (= 100% – FPR) were also performed. The positive predictive value (PPV) was determined as the number of correct positive predictions among all positive predictions from the *in vitro* tests (CP / [CP + FP] × 100%), and the negative predictive value (NPV) as the number of correct negative predictions among all negative predictions from the *in vitro* tests (CN / [CN + FN] × 100%).

Table 2: *In vivo* classifications and *in vitro* test results

Formulation ID	Formulation type[a]	*In vivo* categories			BCOP							EpiOcular-EIT	
					Opacity		Permeability		IVIS		HSI	Relative tissue viability	
		UN GHS	US EPA	Brazil ANVISA	Mean	SD	Mean	SD	Mean[b]	SD	Median	Mean[c]	Inter-tissue difference
1	EC	1	I	I	34.7	2.1	7.97	5.59	**42.7**	7.5	IV	**2.9**	0.1
2	SL	1	I	I	40.7	3.8	65.24	10.68	**106.0**	6.9	IV	**3.0**	0.3
3	EC	1	I	I	22.0	1.9	29.47	6.07	**51.5**	6.3	IV	**3.4**	0.2
4	EC	1	I	I	15.1	2.1	9.19	6.07	**24.3**	6.6	IV	**3.7**	0.5
5	OD	1	I	I	51.7	2.7	31.15	6.82	**82.9**	6.7	III	**5.1**	1.9
6	EC	1	I	I	8.5	3.8	16.47	11.77	**25.0**	15.1	II	**5.2**	1.5
7	EC	1	I	I	18.6	4.9	32.14	7.14	**50.8**	10.5	IV	**5.9**	3.0
8	SL	1	I	I	32.2	2.7	13.76	8.67	**45.9**	6.3	III	**7.6**	0.5
9	SC	1	I	I	7.9	0.4	7.10	3.01	**15.0**	3.4	IV	**7.8**	2.9
10	SL	1	I	I	26.9	8.4	18.14	2.94	**45.1**	9.3	III	**8.4**	3.1
11	EC	1	I	I	3.5	1.6	0.11	0.08	**3.7**	1.5	II	**8.4**	4.0
12	EC	1	I	I	8.9	1.3	7.27	5.50	**16.2**	4.8	n.e.	**9.0**	1.1
13	EC	1	I	I	8.6	1.1	1.30	1.25	**9.9**	0.9	II	**9.0**	1.6
14	SC	1	I	I	24.6	2.3	5.86	1.33	**30.5**	1.2	IV	**11.5**	0.9
15	EC	1	I	I	6.8	3.2	2.41	1.37	**9.2**	4.5	IV	**11.5**	0.5
16	SC	1	I	I	19.5[d]	15.6[d]	0.04[d]	0.64[d]	**19.5[d]**	15.0[d]	I[d]	**12.2**	0.6
17	EC	1	I	I	71.2	4.7	8.64	6.34	**79.8**	10.3	IV	**16.8**	1.3
18	EC	1	I	I	1.5	0.8	0.99	0.82	2.5	0.9	II	**17.4**	4.2
19	SC	1	I	I	2.9	1.1	0.72	0.38	**3.6**	1.4	II	**18.0**	9.3
20	EC	1	I	I	6.9	2.4	0.11	0.10	**7.1**	2.3	I	**25.3**	1.9
21	EC	1	I	I	13.3	2.8	33.01	4.96	**46.3**	6.2	III	**28.9**	7.9
22	EC	2A	II	I	34.1	1.6	48.33	8.60	**82.4**	8.2	n.e.	**3.1**	0.2
23	OD	2A	II	I	68.0	4.4	6.25	1.83	**74.3**	6.1	III	**3.7**	0.4
24	EC	2A	II	I	15.2	2.9	18.52	5.20	**33.7**	7.6	IV	**5.0**	0.7
25	SC	2A	II	I	6.6	2.8	6.51	2.14	**13.1**	4.5	III	**6.0**	4.5

HSI = histological score of irritation; IVIS = in vitro *irritation score; N.cl. = not classified; n.e. = not able to be evaluated due to technical artifacts; SD = standard deviation.*
[a]The formulation types used in this study comprised: CS, capsule suspension; EC, emulsifiable concentrate; FS, flowable concentrate for seed treatment; OD, oil dispersion; SC, suspension concentrate (= flowable concentrate); SE, suspo-emulsion; SL, soluble concentrate; TK, technical concentrate; ZC, a mixed formulation of CS and SC.
[b]Mean IVIS values with IVIS > 55 are in bold type with grey shading; those with 3.0 < IVIS ≤ 55 are in bold type; and values with IVIS ≤ 3 are in normal type writing.
[c]Mean relative viability with relative viability ≤ 60% are in bold type; relative viability < 60 are in normal type.
[d]Values derived from only two corneas.

Table 2: continued

Formulation ID	Formulation type[a]	*In vivo* categories			BCOP							EpiOcular-EIT	
					Opacity		Permeability		IVIS		HSI	Relative tissue viability	
		UN GHS	US EPA	Brazil ANVISA	Mean	SD	Mean	SD	Mean[b]	SD	Median	Mean[c]	Inter-tissue difference
26	EC	2A	III	I	8.9	3.7	1.82	1.21	**10.7**	3.9	n.e.	**6.8**	5.3
27	CS	2A	II	I	9.8	1.3	1.79	1.38	**11.6**	1.3	II	**9.0**	1.0
28	EC	2A	II	I	5.1	1.1	0.11	0.12	**5.2**	1.2	II	**13.6**	4.0
29	SC	2A	II	I	11.8	2.8	–0.30	0.33	**11.5**	3.0	II	**14.5**	2.3
30	EC	2A	II	I	6.0	1.3	0.49	0.32	**6.5**	1.4	IV	**16.3**	1.1
31	EC	2A	II	I	13.3	1.6	2.97	1.14	**16.3**	1.4	n.e.	**16.7**	0.9
32	SC	2A	II	I	1.9	0.9	0.84	0.96	2.8	1.8	II	**18.8**	5.8
33	EC	2A	II	I	–0.3	0.2	0.76	0.54	0.4	0.6	II	**19.1**	0.5
34	EC	2A	II	I	–0.4	1.3	2.79	3.19	2.4	2.3	II	**21.5**	2.7
35	SC	2A	III	I	5.0	3.1	–0.02	0.03	**5.0**	3.0	n.e.	**46.0**	4.0
36	SC	2A	III	I	3.0	0.3	0.03	0.03	**3.0**	0.4	I	**57.5**	0.6
37	SE	2A	III	I	1.4	2.3	0.28	0.49	1.6	2.1	II	61.9	1.5
38	SC	2A	III	I	10.6	3.6	0.25	0.32	**10.9**	3.3	I	65.9	16.5
39	SE	2A	II	I	9.2	2.6	0.00	0.02	**9.2**	2.6	I	68.2	8.6
40	CS	2A	III	I	12.4	1.4	1.64	0.93	**14.1**	2.0	II	111.3	11.4
41	EC	2B	III	I	62.8	7.0	6.98	2.60	**69.7**	8.7	III	**2.8**	0.2
42	EC	2B	III	I	6.9	1.9	0.97	0.52	**7.8**	2.1	III	**7.3**	2.3
43	SC	2B	III	I	5.6	3.1	2.13	0.04	**7.7**	3.1	II	**9.5**	3.1
44	EC	2B	III	I	6.0	1.5	0.12	0.07	**6.2**	1.5	II	**10.0**	0.2
45	EC	2B	III	I	3.3	2.9	57.98	10.26	**61.3**	13.1	n.e.	**13.4**	4.2
46	EC	2B	III	I	4.2	0.3	0.06	0.07	**4.2**	0.3	II	**13.6**	8.2
47	EC	2B	III	I	5.2	0.9	0.23	0.03	**5.5**	0.9	II	**14.9**	12.0
48	EC	2B	III	I	10.9	2.7	1.55	1.01	**12.4**	2.3	n.e.	**16.6**	7.6
49	EC	2B	III	I	13.3	1.7	1.96	1.23	**15.3**	2.8	IV	**20.3**	0.2
50	SC	2B	III	I	3.7	1.7	0.05	0.04	**3.8**	1.7	II	**24.3**	7.3
51	EC	2B	III	I	1.4	1.1	2.83	2.11	**4.2**	1.6	I	**30.0**	8.6
52	EC	2B	III	I	8.9	2.2	0.24	0.14	**9.2**	2.2	II	**30.3**	3.5
53	SC	2B	III	I	2.8	0.3	0.10	0.07	2.9	0.3	II	**32.6**	7.4
54	SC	2B	III	II	–0.5	0.9	0.07	0.03	–0.4	0.9	I	60.2	1.3
55	EC	N.cl.	III	I	14.6	1.2	2.19	1.85	**16.8**	1.1	n.e.	**6.1**	0.7

Table 2: continued

Formulation ID	Formulation type[a]	*In vivo* categories			BCOP							EpiOcular-EIT	
					Opacity		Permeability		IVIS		HSI	Relative tissue viability	
		UN GHS	US EPA	Brazil ANVISA	Mean	SD	Mean	SD	Mean[b]	SD	Median	Mean[c]	Inter-tissue difference
56	CS	N.cl.	III	I	12.3	1.9	1.99	1.23	**14.3**	3.0	II	**16.6**	2.5
57	SC	N.cl.	III	I	7.2	1.0	7.10	1.18	**14.3**	2.1	II	**17.0**	9.5
58	EC	N.cl.	III	I	12.9	0.7	0.94	0.86	**13.8**	1.4	II	**17.0**	2.0
59	EC	N.cl.	III	I	3.9	4.0	40.43	7.72	**44.3**	5.5	n.e.	**17.3**	0.4
60	TK	N.cl.	III	III	2.4	1.1	3.86	0.40	6.2	0.8	II	**21.0**	6.3
61	SL	N.cl.	IV	IV	1.6	0.2	0.19	0.06	1.8	0.2	II	**22.3**	3.6
62	SC	N.cl.	III	I	5.8	2.9	0.05	0.03	5.9	2.9	I	**23.2**	13.8
63	EC	N.cl.	III	I	0.8	0.3	–0.94	0.07	–0.1	0.2	I	**40.1**	6.4
64	CS	N.cl.	III	I	7.6	1.2	0.09	0.03	7.7	1.2	II	**46.4**	3.4
65	SL	N.cl.	IV	IV	1.3	1.0	0.24	0.22	1.5	0.8	II	**52.6**	11.5
66	SE	N.cl.	III	III	8.4	3.0	–1.22	0.11	7.2	3.1	I	**56.9**	12.7
67	SC	N.cl.	III	I	0.8	0.8	1.86	1.91	2.6	1.5	I	61.5	10.9
68	SC	N.cl.	III	III	0.0	0.8	0.74	0.48	0.8	0.4	II	64.8	0.1
69	SE	N.cl.	III	I	5.7	2.8	0.58	0.71	6.3	3.0	I	66.5	5.8
70	SC	N.cl.	IV	IV	5.2	4.5	0.77	1	6.0	5.2	I	67.8	13.1
71	EC	N.cl.	III	I	1.8	0.5	0.05	0.06	1.8	0.5	I	68.5	4.9
72	SC	N.cl.	III	III	–3.4	0.5	–0.01	0.06	–3.4	0.5	I	69.1	1.1
73	ZC	N.cl.	IV	IV	–0.4	1.5	0.08	0.07	–0.3	1.6	I	71.2	14.9
74	SC	N.cl.	III	III	–0.1	0.4	0.02	0.03	–0.1	0.4	0	76.9	9.8
75	SC	N.cl.	III	III	0.7	0.7	0.38	0.61	1.1	0.9	0	78.4	13.4
76	ZC	N.cl.	IV	IV	0.2	0.3	0.02	0.02	0.2	0.3	0	79.2	10.9
77	SL	N.cl.	IV	IV	2.0	2.3	0.09	0.20	2.1	2.1	I	84.1	1.8
78	SC	N.cl.	IV	IV	0.4	0.8	0.37	0.63	0.8	1.4	0	84.9	2.9
79	SC	N.cl.	IV	IV	2.5	3.2	0.03	0	2.6	3.2	0	87.0	8.1
80	SL	N.cl.	IV	IV	1.6	0.6	0.03	0.01	1.6	0.6	0	87.0	9.1

HSI = histological score of irritation; IVIS = in vitro *irritation score; N.cl. = not classified; n.e. = not able to be evaluated due to technical artifacts; SD = standard deviation.*
[a]The formulation types used in this study comprised: CS, capsule suspension; EC, emulsifiable concentrate; FS, flowable concentrate for seed treatment; OD, oil dispersion; SC, suspension concentrate (= flowable concentrate); SE, suspo-emulsion; SL, soluble concentrate; TK, technical concentrate; ZC, a mixed formulation of CS and SC.
[b]Mean IVIS values with IVIS > 55 are in bold type with grey shading; those with 3.0 < IVIS ≤ 55 are in bold type; and values with IVIS ≤ 3 are in normal type writing.
[c]Mean relative viability with relative viability ≤ 60% are in bold type; relative viability < 60 are in normal type.
[d]Values derived from only two corneas.

Table 2: continued

Formulation ID	Formulation type[a]	*In vivo* categories			BCOP							EpiOcular-EIT	
					Opacity		Permeability		IVIS		HSI	Relative tissue viability	
		UN GHS	US EPA	Brazil ANVISA	Mean	SD	Mean	SD	Mean[b]	SD	Median	Mean[c]	Inter-tissue difference
81	SE	N.cl.	IV	IV	2.6	1.6	0.06	0.02	2.6	1.7	0	87.2	3.4
82	SC	N.cl.	IV	IV	0.5	0.9	0.08	0.06	0.6	0.9	0	90.3	7.5
83	SC	N.cl.	III	III	2.3	1.4	0.01	0.07	2.3	1.3	0	94.5	3.5
84	SC	N.cl.	IV	IV	–1.2	1.4	0.00	0.05	–1.2	1.4	0	95.1	8.0
85	SC	N.cl.	III	III	–1.2	1.3	–0.02	0.03	–1.2	1.3	0	95.5	7.3
86	SC	N.cl.	IV	IV	1.6	1.6	0.02	0.08	1.6	1.7	0	95.5	1.1
87	FS	N.cl.	IV	IV	0.8	1.3	0.03	0.00	0.8	1.3	0	95.7	6.8
88	SC	N.cl.	IV	IV	0.6	0.9	0.11	0.07	0.7	1.0	0	97.4	4.2
89	SC	N.cl.	III	I	2.4	0.9	–0.02	0.02	2.4	0.9	0	98.1	1.0
90	SC	N.cl.	IV	IV	–0.2	2.6	–0.02	0.00	–0.2	2.6	0	102.1	0.7
91	SC	N.cl.	IV	IV	3.4	0.5	–0.02	0.25	3.4	0.5	0	105.0	0.7
92	SC	N.cl.	IV	IV	–1.6	0.7	0.05	0.09	–1.5	0.8	0	105.4	4.8
93	FS	N.cl.	IV	IV	–0.2	0.6	0.01	0.36	–0.2	0.4	0	108.1	4.3
94	SC	N.cl.	III	III	2.2	1.0	0.09	0.06	2.3	1.0	I	109.8	13.8
95	SC	N.cl.	IV	IV	0.9	1.1	-0.01	0.05	0.9	1.1	I	111.3	11.4
96	SC	N.cl.	III	III	–0.3	0.6	0.12	0.05	–0.2	0.5	I	115.0	5.3
97	SE	N.cl.	III	III	–3.1	4.8	0.63	0.67	–2.4	4.1	0	120.5	1.4

HSI = histological score of irritation; IVIS = in vitro *irritation score; N.cl. = not classified; n.e. = not able to be evaluated due to technical artifacts; SD = standard deviation.*
[a]The formulation types used in this study comprised: CS, capsule suspension; EC, emulsifiable concentrate; FS, flowable concentrate for seed treatment; OD, oil dispersion; SC, suspension concentrate (= flowable concentrate); SE, suspo-emulsion; SL, soluble concentrate; TK, technical concentrate; ZC, a mixed formulation of CS and SC.
[b]Mean IVIS values with IVIS > 55 are in bold type with grey shading; those with 3.0 < IVIS ≤ 55 are in bold type; and values with IVIS ≤ 3 are in normal type writing.
[c]Mean relative viability with relative viability ≤ 60% are in bold type; relative viability < 60 are in normal type.
[d]Values derived from only two corneas.

Results

In this study, we have used *in vivo* eye irritation data on 97 agrochemical formulations to assess the predictive capacity of the BCOP test including corneal histopathology and the EpiOcular-EIT.

Classification differences based on *in vivo* data

Tables 3a and 3b show a comparison of the *in vivo* classifications of the formulations tested in this study according to the UN GHS (12), US EPA (13) and Brazil ANVISA (14) classification schemes. The 21 agrochemical formulations assigned to UN GHS Category 1 were identical to those assigned to US EPA Category I, while those in UN GHS Categories 2A, 2B and No Category were not always congruent with those in US EPA Classes II, III, IV (Table 3a). In summary, US EPA Categories I, II, III and IV were 70% congruent with UN GHS Categories 1, 2A, 2B, and No Category. The 21 agrochemical formulations assigned to UN GHS Category 1 were also assigned to Brazil ANVISA Toxicity Class I; however, 44 additional agrochemicals (assigned to UN GHS Categories 2A, 2B or No Category) were also assigned to Brazil ANVISA Toxicity Class I (Table 3b). In summary, Brazil ANVISA Toxicity Classes I, II, III and IV were 42% congruent with UN GHS Categories 1, 2A, 2B and No Category.

In vitro data

The results obtained in the EpiOcular-EIT and the BCOP test including corneal histopathology, as well as the *in vivo* classification, are summarised in Table 2.

UN GHS classification

BCOP and corneal histopathology

In the BCOP prediction model, the median IVIS values were 25.0 for UN GHS Category 1 formulations (with mean IVIS values $2.5 \leq \text{IVIS} \leq 106.0$), 10.7 for UN GHS Category 2A formulations (with mean IVIS values $0.4 \leq \text{IVIS} \leq 82.9$), 6.9 for UN GHS Category 2B formulations (with mean IVIS values $0.4 \leq \text{IVIS} \leq 69.7$), and 1.6 for not classified formulations (UN GHS No Category; with mean IVIS values $3.4 \leq \text{IVIS} \leq 44.3$).

According to the BCOP prediction model with a cut-off of IVIS > 55 for the identification of severe

Table 3a: A comparison of US EPA and UN GHS classifications of the tested agrochemical formulations

		UN GHS classification				
		1	**2A**	**2B**	**No**	∑
US EPA Category	**I**	**21**	0	0	0	21
	II	0	**13**	0	0	13
	III	0	6	**14**	23	43
	IV	0	0	0	**20**	20
	∑	21	19	14	43	**97**

Table 3b: A comparison of ANVISA and UN GHS classifications of the tested agrochemical formulations

		UN GHS classification				
		1	**2A**	**2B**	**No**	∑
Brazil ANVISA toxicity class	**I**	**21**	19	13	12	65
	II	0	**0**	1	1	1
	III	0	0	**0**	11	11
	IV	0	0	0	**20**	20
	∑	21	19	14	43	**97**

Table 4: UN GHS classification based on the BCOP test

		In vivo				
		No cat.	**Cat. 2B**	**Cat. 2A**	**Cat. 1**	**∑**
BCOP test	**3 ≤ IVIS**	31	2	4	1	38
	3 < IVIS ≤ 55	12	10	13	17	52
	IVIS > 55	0	2	2	3	7
	∑	43	14	19	21	**97**

ocular irritants (UN GHS Category 1), as described for single component test substances and formulations (3) (Tables 4 and 9, Figure 1), 75 out of 97 formulations were predicted correctly as either Category 1 or not Category 1 (77% accuracy). Of 76 UN GHS Category 2A, 2B or not classified formulations, four were over-predicted (5% false positives), and 18 out of 21 UN GHS Category 1 formulations were under-predicted (86% false negatives). Of 21 UN GHS Category 1 formulations, three were correctly predicted as severe irritants (14% sensitivity), and 72 out of 76 UN GHS Category 2A, 2B or not classified formulations were correctly predicted as non-irritants (95% specificity). The NPV was 80% (72/90), and the PPV was 43% (3/7).

According to the BCOP prediction model with a cut-off of IVIS ≤ 3 for the identification of ocular non-irritants (UN GHS No Category), as described for single component test substances and formulations (3), 78 out of 97 formulations were predicted correctly as non-irritants (UN GHS No Category) or ocular irritants (UN GHS Categories 1, 2A or 2A), resulting in 80% accuracy. Of 43 not classified formulations (UN GHS No Category), 12 were over-predicted (28% false positives) and seven out of 54 UN GHS Category 1, 2A or 2B formulations were under-predicted (13% false negatives). Of 54 UN GHS Category 1, 2A, or 2B formulations, 47 were correctly predicted as severe irritants (87% sensitivity), 31 out of 43 not classified formulations (UN GHS No Category) were correctly predicted as non-irritants (72% specificity). The NPV was 82% (31/38) and the PPV was 80% (47/59).

In addition to the opacity and permeability endpoints, corneal histopathology was evaluated after the assessment of the two former endpoints. By using corneal histopathology, the HSI score for UN GHS Category 1 formulations resulted in a median of III (with individual median HSI values of I ≤ HSI ≤ IV), for UN GHS Category 2A and 2B formulations in a median of II (with individual median HSI values of I ≤ HSI ≤ IV), and for not classified formulations (UN GHS No Category) a median of I (with individual median HSI values of 0 ≤ HSI ≤ III). A median HSI of IV was used as a predictor of severe ocular irritants. As shown in Tables 5 and 10 and Figure 1, a total of 74 out of 97 formulations were predicted correctly (76% accuracy), including the two formulations correctly predicted by the IVIS. Of 76 UN GHS Category 2A, 2B or not classified formulations (UN GHS No Category), three were over-predicted (4% false positives) and 11 out of 21 UN GHS Category 1 formulations were under-predicted (52% false negatives).

EpiOcular-EIT

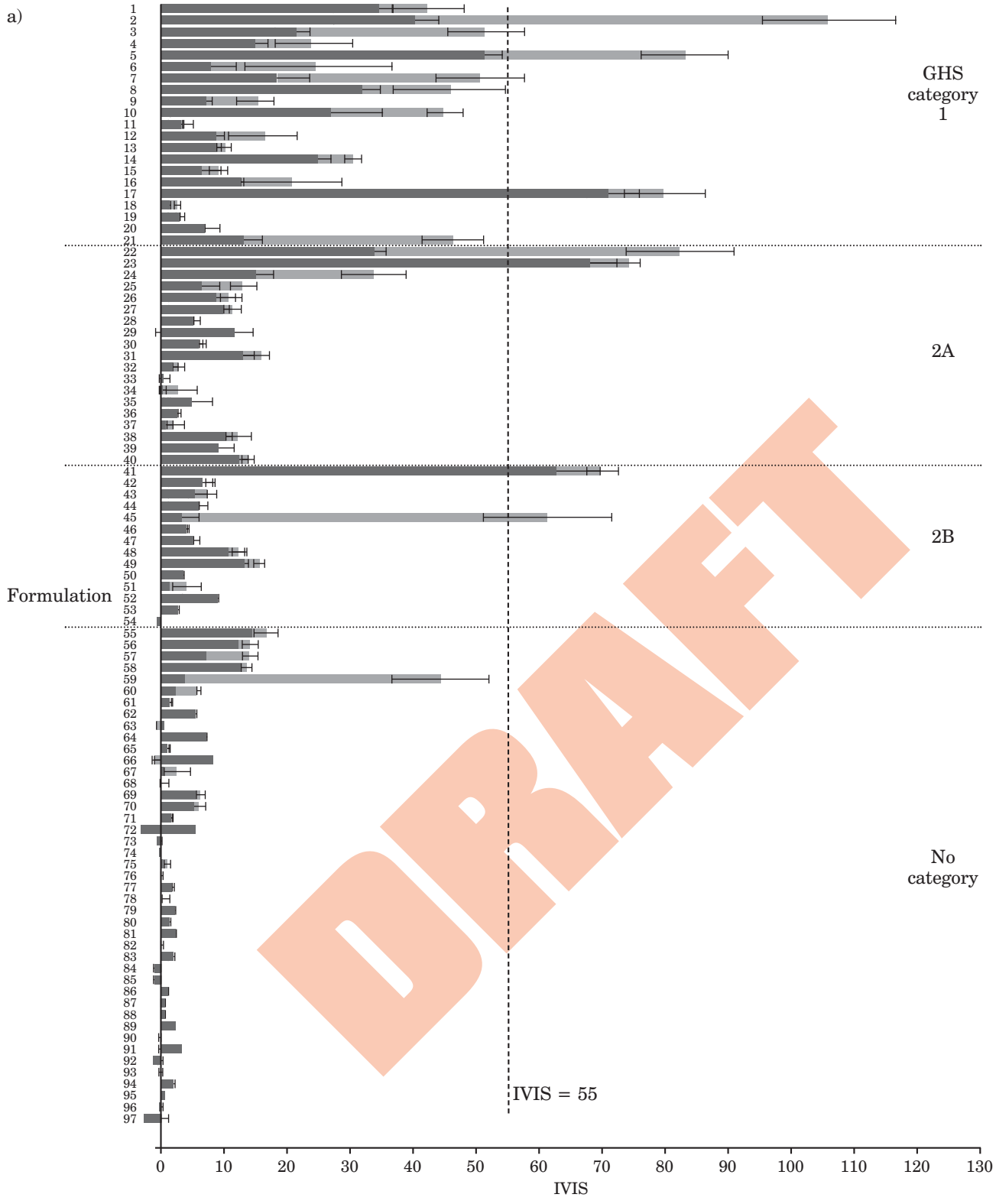
In the EpiOcular-EIT, the median relative viability for the different classifications was: 8.4% for

Table 5: UN GHS classification based on the BCOP HSI test

		In vivo				
		No cat.	**Cat. 2B**	**Cat. 2A**	**Cat. 1**	**∑**
BCOP HSI test	**0**	20	0	0	0	20
	I	13	2	3	2	20
	II	8	7	8	5	28
	III	0	2	2	4	8
	IV	0	1	2	9	12
	n.e.	2	2	4	1	9
	∑	43	14	19	21	**97**

n.e. = not able to be evaluated due to technical artifacts.

Figure 1: *In vitro* eye irritation data sorted according to UN GHS classification of Draize test data



Agrochemical formulations assigned to UN GHS Categories 1, 2A, 2B, and not classified (UN GHS No Category) according to the in vivo *Draize rabbit eye irritation test were tested in the BCOP test by using the endpoints opacity, permeability and corneal histology, and in the EpiOcular™ EIT. a) Mean BCOP IVIS (opacity + 15 × permeability); b) median BCOP HSI, and c) mean relative tissue viability of two EpiOcular™ tissues plus inter-tissue difference. n.e. = unable to be evaluated due to technical artifacts.*

= opacity; = 15× permeability.

Figure 1: continued

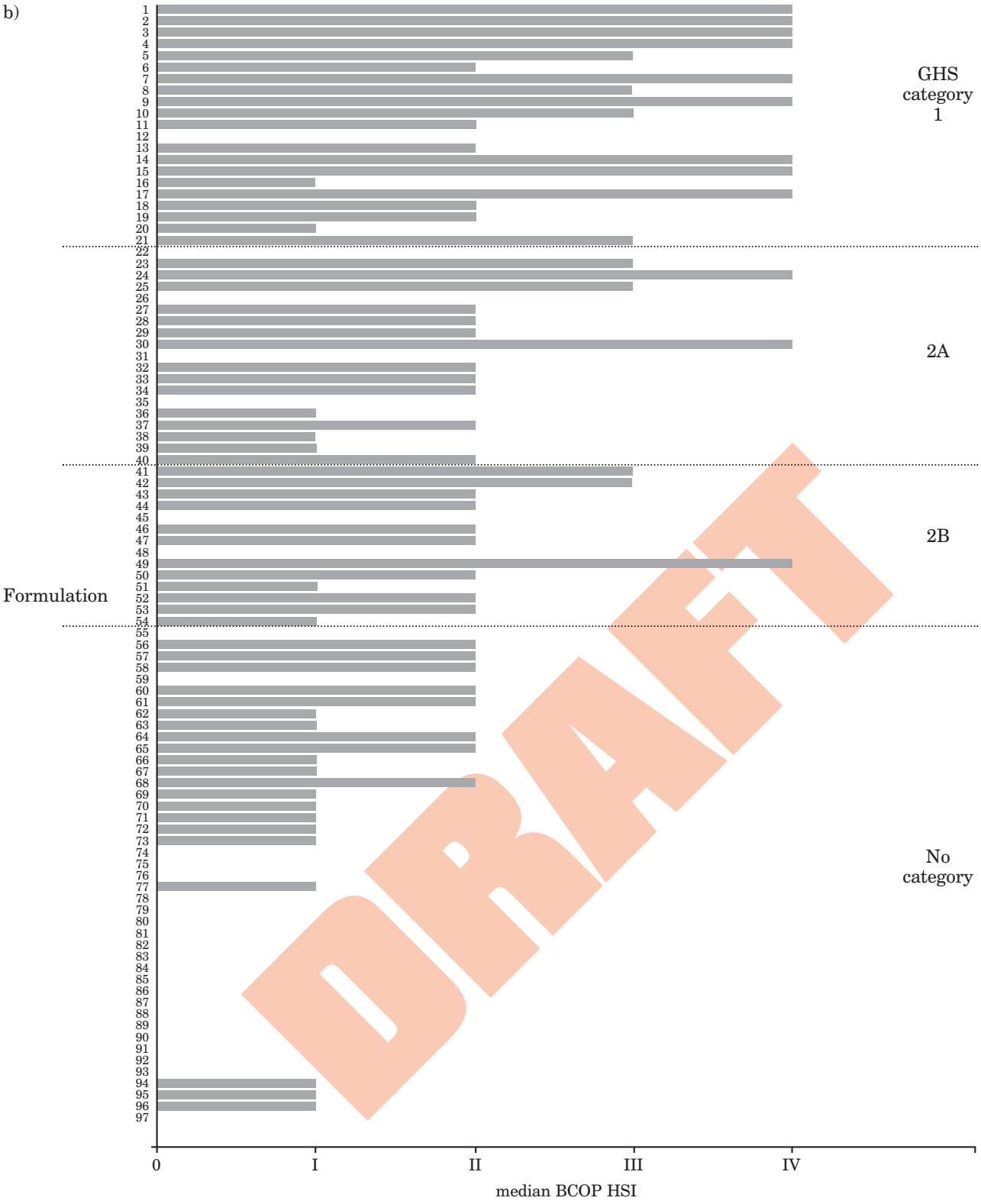
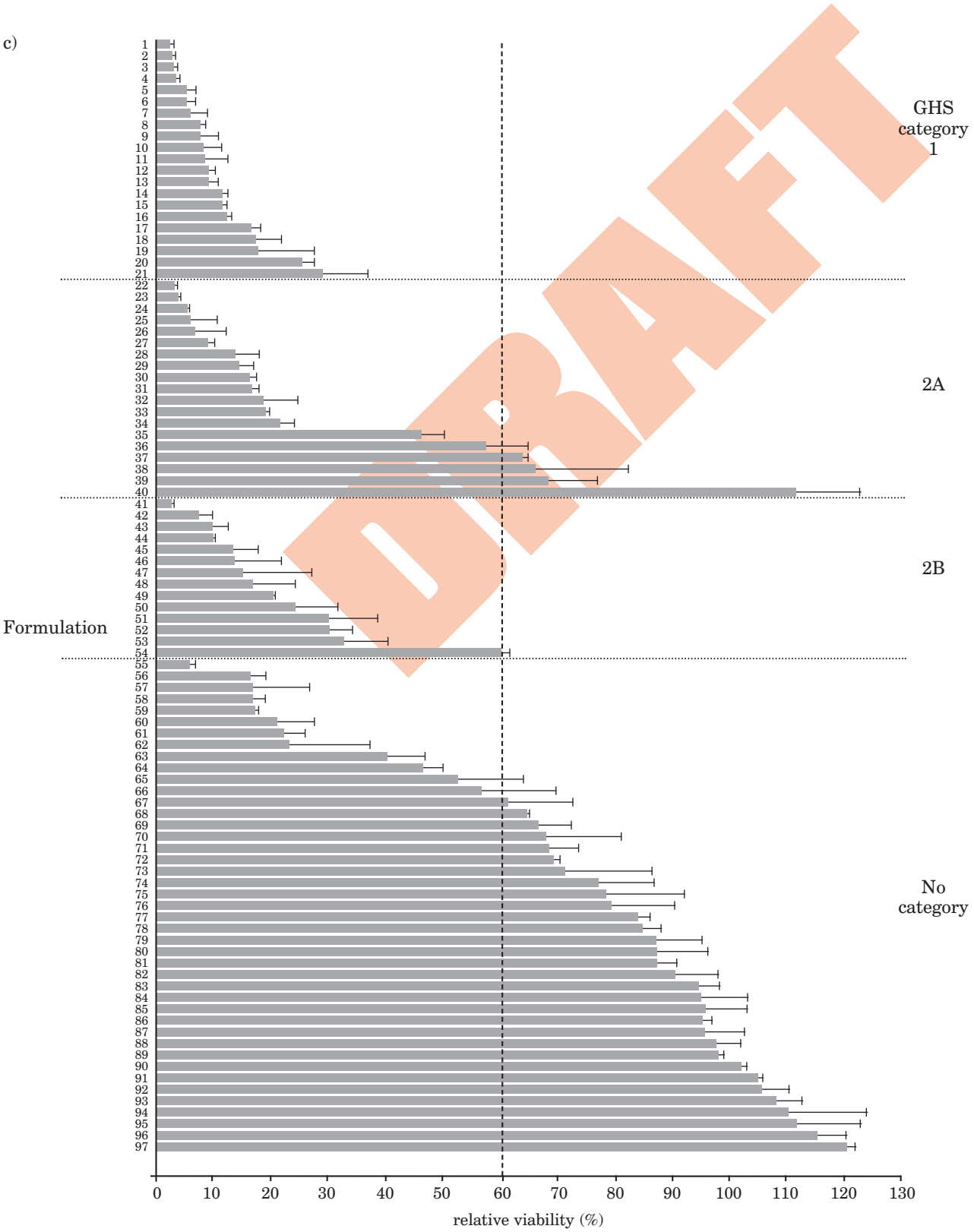


Figure 1: continued



Agrochemical formulations assigned to UN GHS Categories 1, 2A, 2B, and not classified (UN GHS No Category) according to the in vivo *Draize rabbit eye irritation test were tested in the BCOP test by using the endpoints opacity, permeability and corneal histology, and in the EpiOcular™ EIT. a) Mean BCOP IVIS (opacity + 15 × permeability); b) median BCOP HSI, and c) mean relative tissue viability of two EpiOcular™ tissues plus inter-tissue difference. n.e. = unable to be evaluated due to technical artifacts.*

Table 6: UN GHS classification based on the EpiOcular-EIT

		In vivo				
		No cat.	**Cat. 2B**	**Cat. 2A**	**Cat. 1**	
EpiOcular-EIT	**Rel. viability > 60%**	31	1	4	0	36
	Rel. viability ≤ 60%	12	13	15	21	61
		43	14	19	21	**97**

Table 7: Predictive capacity of the EpiOcular-EIT to predict ocular non-irritant agrochemical formulations according to UN GHS (relative viability cut-off 60%) by formulation type

	All formulation types	**EC formulations**	**SC formulations**
Sensitivity	91% (49/54)	100% (31/31)	86% (12/14)
Specificity	72% (31/43)	20% (1/5)	91% (21/23)
Accuracy	83% (80/97)	89% (32/36)	89% (33/37)
False negatives	9% (5/54)	0% (0/31)	14% (2/14)
False positives	28% (12/43)	80% (4/5)	9% (2/23)
Positive predictive value	80% (49/61)	89% (31/35)	86% (12/14)
Negative predictive value	86% (31/36)	100% (1/1)	91% (21/23)

For the purpose of this analysis, the formulations considered ocular non-irritants according to the animal data were: for UN GHS, agrochemical formulations that were not classified (UN GHS No category); for US EPA, those that were assigned to US EPA Category IV; and for Brazil ANVISA, those that were assigned to Brazil ANVISA Toxicity Class IV.

UN GHS Category 1 formulations (with mean relative viability values of 2.9% ≤ relative viability ≤ 28.9%); 16.7% for UN GHS Category 2A formulations (with mean relative viability values of 3.1% ≤ relative viability ≤ 111.3%); 15.8% for UN GHS Category 2B formulations (with mean relative viability values of 2.8% ≤ relative viability ≤ 60.2%); and 79.2% for UN GHS No Category (with mean relative viability values of 6.1% ≤ relative viability ≤ 120.5%).

According to the EpiOcular-EIT prediction model with a 60% relative viability cut-off for the identification of ocular non-irritants (UN GHS No Category; Tables 6 and 8, Figure 1), 80 out of 97 formulations were predicted correctly (83% accuracy). Of 43 formulations not classified according to UN GHS, 12 were over-predicted (28% false positives). Of 54 UN GHS Category 1, 2A, or 2B formulations, five were under-predicted (9% false negatives). Finally, 31 out of 43 not classified formulations according to UN GHS were correctly predicted as non-irritants (72% specificity), and of the 54 UN GHS Category 1, 2A, or 2B formulations, 49 were correctly predicted as irritants (91% sensitivity), but without a distinction between irritant UN GHS Categories 1, 2A or 2B. The NPV was 86% (31/36) and the PPV 80% (49/61).

Analysis of Formulation Types in the EpiOcular-EIT: The agrochemical formulations tested in this study comprised nine formulation types (CS, EC, FS, OD, SC, SE, SL, TK, and ZC). Of those, only two types, namely EC and SC, were represented by 30 or more formulations, and the predictive capacity of the EpiOcular-EIT has been analysed for those separately (Table 7). In summary, the predictive capacity of the EpiOcular-EIT for SC-type formulations was similar to the predictivity for all types of formulations assessed. The majority of EC formulations tested (31 out of 36) were classified as UN GHS Categories 1 or 2 *in vivo*. Of the five *in vivo* non-irritant EC formulations, one was correctly predicted in the EpiOcular-EIT, resulting in 20% specificity.

Comparison of the predictive capacity of the EpiOcular-EIT and the BCOP test including corneal histopathology, within the UN GHS, US EPA and Brazil ANVISA classification schemes

The predictive capacity of the EpiOcular-EIT and the BCOP test including corneal histopathology for the UN GHS, US EPA and Brazil ANVISA classi-

Table 8: Predictive capacity of the EpiOcular-EIT to predict ocular non-irritant agrochemical formulations according to different classification schemes (relative viability cut-off 60%)

	UN GHS	US EPA	Brazil ANVISA
Sensitivity	91% (49/54)	77% (59/77)	77% (59/77)
Specificity	72% (31/43)	90% (18/20)	90% (18/20)
Overall accuracy	83% (80/97)	79% (77/97)	79% (77/97)
False negatives	9% (5/54)	23% (18/77)	23% (18/77)
False positives	28% (12/43)	10% (2/20)	10% (2/20)
Positive predictive value	80% (49/61)	97% (59/61)	97% (59/61)
Negative predictive value	86% (31/36)	50% (18/36)	50% (18/36)

For the purpose of this analysis, the formulations considered ocular non-irritants according to the animal data were: for UN GHS, agrochemical formulations that were not classified (UN GHS No category); for US EPA, those that were assigned to US EPA Category IV; and for Brazil ANVISA, those that were assigned to Brazil ANVISA Toxicity Class IV.

fication schemes, is summarised in Tables 8–10. The EpiOcular-EIT was found to have the highest sensitivity when classifying the *in vivo* data according to UN GHS, while specificity was higher when comparing to *in vivo* data classified according to US EPA and Brazil ANVISA. The overall accuracy was in the 80% range for all classification schemes (Table 8).

The sensitivity of the standard BCOP test for the most severe categories was unacceptably low (11–14%), while specificity was above 90% for all the classification schemes. The overall accuracy was in the 75% range for the UN GHS and US EPA classification schemes, but below 50% for that of Brazil ANVISA (Table 9).

The sensitivity of the corneal histopathology for the most severe categories was also unacceptably low, albeit higher than when relying solely on the IVIS score from the standard BCOP test; the specificity was above 85% for all classification schemes. The overall accuracy was 76% for the UN GHS and US EPA classification schemes, but below 50% for that of Brazil ANVISA (Table 10).

Discussion

In vivo data and classification considerations

When establishing and validating *in vitro* methods to replace *in vivo* tests, the quality of the reference *in vivo* data is crucial. The variability within an *in vivo* test may even lead to misclassification (23). Hence, the variability of the *in vivo* test and the consequent uncertainty of the result influences the overall accuracy of a test outcome, and may therefore hamper the successful validation of *in vitro* methods (24). Recently, Adriaens *et al.* (23) have underlined the importance of understanding the drivers of (UN GHS) classification, and have described irreversibility as the major driver for UN GHS Category 1, while corneal opacity and conjunctival redness were the major drivers for the

Table 9: Predictive capacity of the BCOP test to predict severe ocular irritation according to different classification schemes (IVIS cut-off for severe irritants IVIS > 55)

	UN GHS	US EPA	Brazil ANVISA
Sensitivity	14% (3/21)	14% (3/21)	11% (7/65)
Specificity	95% (72/76)	95% (72/76)	100% (32/32)
Overall accuracy	77% (75/97)	77% (75/97)	40% (39/97)
False negatives	86% (18/21)	86% (18/21)	89% (58/65)
False positives	5% (4/76)	5% (4/76)	0% (0/32)
Positive predictive value	43% (3/7)	43% (3/7)	100% (7/7)
Negative predictive value	80% (72/90)	80% (72/90)	36% (32/90)

For the purpose of this analysis, the formulations considered severe ocular irritants according to the animal data were: for UN GHS, agrochemical formulations that were assigned to UN GHS Category 1; for US EPA, those that were assigned to US EPA Category I; and for Brazil ANVISA, those that were assigned to Brazil ANVISA Toxicity Class I.

Table 10: Predictive capacity of the corneal histopathology HSI test to predict severe ocular irritation according to different classification schemes (HSI cut-off IV)

	UN GHS	US EPA	Brazil ANVISA
Sensitivity	43% (9/21)	43% (9/21)	18% (12/65)
Specificity	86% (65/76)	86% (65/76)	100% (32/32)
Overall accuracy	76% (74/97)	76% (74/97)	45% (44/97)
False negatives	52% (11/21)	52% (11/21)	68% (44/65)
False positives	4% (3/76)	4% (3/76)	0% (0/32)
Not able to be evaluated due to technical artifacts	9% (9/97)	9% (9/97)	9% (9/97)

For the purpose of this analysis, the formulations considered severe ocular irritants according to the animal data were: for UN GHS, agrochemical formulations that were assigned to UN GHS Category 1; for US EPA, those that were assigned to US EPA Category I; and for Brazil ANVISA, those that were assigned to Brazil ANVISA Toxicity Class I.

GHS Category 2 classification of chemicals. The *in vitro* tests used in this study were either organotypic (BCOP test) or cytotoxicity/cell function-based assays (EpiOcular-EIT). Both are single short-time exposure *in vitro* methods, and neither test assesses the reversibility of the effects. Further, while the EpiOcular-EIT has been described as correctly predicting all the types of ocular injuries observed *in vivo*, including those on the conjunctiva, cornea and iris (5), the BCOP test does not cover all possible *in vivo* ocular effects such as conjunctiva and iris injuries. Here, we present a correlation analysis of the data from the EpiOcular-EIT and the BCOP test including corneal histopathology, to Draize *in vivo* data, for 97 agrochemical formulations, according to the different classification schemes.

Depending on which type of effect is regarded as most severe — i.e. irreversibility or persistence of significant corneal opacity and/or iritis for the UN GHS and US EPA classifications, and corneal opacity in the Brazil ANVISA classification — different regulatory classification schemes can result in different classifications based on the same *in vivo* data. Those differences may be quite significant: based on the same *in vivo* data, 21 agrochemical formulations were classified as Category 1 by the UN GHS scheme, while 65 were assigned to Toxicity Class I by the Brazil ANVISA scheme; those assigned to UN GHS Category 1 were concordant to those assigned to US EPA Class I. Taking into account that *in vivo* data are used as a reference when developing and/or validating *in vitro* methods, the prediction models used for the different classification schemes certainly need adaptation for the individual classification schemes.

Test systems

During the last decades, significant progress in the field of replacement methods for ocular irritation has been made, and several methods have gained, or are close to gaining, regulatory acceptance. We present here a simple correlation analysis of *in vivo* data, classified according to three classification schemes, with the BCOP test including corneal histopathology and the EpiOcular-EIT.

The BCOP test has undergone formal validation, and has been accepted for the identification of substances and mixtures inducing serious eye damage and for those that do not require classification for eye irritation or serious eye damage. The test is known to produce false positive predictions for alcohols and ketones, and false negative predictions for solids, when used for the identification of substances inducing serious eye damage. The specific applicability or non-applicability of the BCOP test protocol described in OECD TG 437 for agrochemical formulations has not yet been described. In the present study, the BCOP test was performed according to the protocol for liquids described in OECD TG 437 (3), with the intention of identifying severe ocular irritant agrochemical formulations (IVIS cut-off 55). For this evaluation, 97 liquid agrochemical formulations with available *in vivo* data were tested in the BCOP test in conjunction with histopathological evaluation. Irrespective of the classification scheme, based on the standard endpoints of opacity and permeability, the BCOP test was not sufficiently sensitive (sensitivity of 11–14% depending on the classification scheme) to predict the highest toxicity classification.

In addition to its use for the testing of chemicals, the BCOP assay is also part of the test battery used in the EPA Antimicrobial Cleaning Product testing programme. Despite the recommendation of the assay, when performed according to the standard protocols in OECD TG 437 with an IVIS of 75 as the cut-off, for the identification of EPA Toxicity Class I antimicrobial cleaning products (25), in the current study, it failed to predict severe ocular irritating agrochemical formulations, even with the less-stringent IVIS cut-off of 55. Histopathology has been described as a useful additional parameter, particularly for the evalua-

tion of the depth of injury (as a potential indirect indicator of reversibility; 3). Indeed, when using histopathology of the bovine corneas with an HSI IV as cut-off for the most severe classification, the sensitivity was significantly higher than with the standard BCOP endpoints. However, the sensitivity of the corneal histopathology used in this study was still considered to be too low (18–43% depending on the classification scheme) to be used as the basis for the hazard assessment. In summary, we recommend against the use of the BCOP protocol as described in OECD TG 437 (3) for the identification of severe ocular irritant agrochemical formulations (GHS Category 1, EPA Toxicity Category I or Brazil ANVISA Toxicity Class I).

The EpiOcular-EIT has undergone formal validation, and an OECD draft TG has been issued for the identification of substances that do not require classification for eye irritation or serious eye damage. No limitations are currently known with regard to the spectrum of chemicals to which the assay is applicable — it is assumed to be applicable to the full spectrum of chemical classes and physicochemical properties (5). A test based on the same tissue model, but evaluating the irritant potential of a test substance based on the exposure time needed to decrease relative viability by 50%, is also part of the test battery used in the EPA Antimicrobial Cleaning Product testing programme (25).

In the data set presented here, five out of 54 (9%) formulations were predicted as false negatives in the EpiOcular-EIT, but none of the five FN formulations was classified *in vivo* as UN GHS Category 1 (four were UN GHS Category 2A and one UN GHS Category 2B), and the effects observed *in vivo* were mostly mild (formulations 37, 38 and 40) or even at the border of classification (formulation 54; data not shown). The percentage of false negatives observed for the EpiOcular-EIT seems comparable to that obtained *in vivo*: based on the within-test variability alone, the rabbit test presents with a 12% probability to misclassify UN GHS Category 2 substances as non-irritants (UN GHS No Category; 23). In addition, four of the five false negative formulations resulted in relative viability values close to the cut-off (values between 60.2% and 68.2% were obtained for formulations 37, 38, 39 and 54). In summary, the sensitivity, specificity and overall accuracy values obtained in this study (91%, 72% and 83%) for the prediction of UN GHS are very close to those resulting from the formal validation study of the EpiOcular-EIT, which tested 113 chemicals with an overall accuracy of 80%, sensitivity of 96% and specificity of 63% (22). Therefore, we propose that, generally, liquid agrochemical formulations should be considered to be within the applicability domain of the EpiOcular-EIT.

Summary and Conclusion

In this study, 97 liquid agrochemical formulations that were previously classified according to three different schemes based on *in vivo* data, have been assessed in the EpiOcular-EIT and the BCOP test including corneal histopathology. The diverse criteria for categorisation in the existing classification schemes resulted in significantly different designations. In this sense, the Brazil ANVISA classification was particularly divergent from the UN GHS and US EPA classification schemes, which were more similar. Opacity and permeability determined according to the standard BCOP test protocol for liquids, and the resulting IVIS, were not useful in the prediction of severe ocular irritant agrochemical formulations according to the classification schemes of the UN GHS, US EPA or Brazil ANVISA. Corneal histopathology was a better predictor for severe eye irritation of the agrochemical formulations, but still not with an acceptable sensitivity. Hence, we conclude that the BCOP test according to the standard protocol for liquids is not suitable for the testing of liquid agrochemical formulations, and protocol refinements are necessary to putatively render the assay useful for this type of test substances. The relative viability in the EpiOcular-EIT was a useful predictor to distinguish irritant (Categories 1, 2A and 2B) and non-irritant formulations (No Category), as determined by UN GHS. The predictive capacity of the EpiOcular-EIT for the 97 agrochemical formulations was comparable to that obtained in the formal validation exercise with a diverse group of ocular irritants and non-irritants. We therefore conclude that the liquid protocol for the EpiOcular-EIT is applicable to agrochemical formulations.

Acknowledgements

We would like to thank the Laboratory for Applied Alternative Methods at BASF SE for their excellent technical assistance, and Dr Torsten Knieriem for his formulation expertise and support in the first phase of this project.

Received 03.02.15; received in final form 28.04.15; accepted for publication 28.04.15.

References

1. OECD (2002). *OECD Guidelines for the Testing of Chemicals, No. 405: Acute Eye Irritation/Corrosion*, 14pp. Paris, France: Organisation for Economic Co-operation and Development.
2. Curren, R.D. & Harbell, J.W. (2002). Ocular safety: A silent (*in vitro*) success story. *ATLA* **30**, Suppl. 2, 69–74.
3. OECD (2013). *OECD Guidelines for the Testing of*

Chemicals, Test No. 437: Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage, 27pp. Paris, France: Organisation for Economic Co-operation and Development.

4. OECD (2013). *OECD Guidelines for the Testing of Chemicals, Test No. 438: Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage*, 20pp. Paris, France: Organisation for Economic Co-operation and Development.
5. OECD (2014). *OECD Guidelines for the Testing of Chemicals, Draft Proposal for a New Test Guideline: Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*, 25pp. Paris, France: Organisation for Economic Co-operation and Development.
6. UN (2011). *Globally Harmonized System of Classification and Labelling of Chemicals (GHS): Fourth Revised Edition*. Geneva, Switzerland: United Nations Economic Commission for Europe.
7. Kolle, S.N., Kandarova, H., Wareing, B., van Ravenzwaay, B. & Landsiedel, R. (2011). In-house validation of the EpiOcular™ eye irritation test and its combination with the bovine corneal opacity and permeability test for the assessment of ocular irritation. *ATLA* **39**, 365–387.
8. Schrage, A., Kolle, S.N., Moreno, M.C., Norman, K., Raabe, H., Curren, R., van Ravenzwaay, B. & Landsiedel, R. (2011). The bovine corneal opacity and permeability test in routine ocular irritation testing and its improvement within the limits of OECD test guideline 437. *ATLA* **39**, 37–53.
9. Prinsen, M.K. (1996). The chicken enucleated eye test (CEET): A practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. *Food & Chemical Toxicology* **34**, 291–296.
10. ICCVAM (2006). *ICCVAM Test Method Evaluation Report:* In Vitro *Test Methods for Detecting Ocular Corrosives and Severe Irritants*, 484pp. Bethesda, MA, USA: National Institutes of Health.
11. Schrage, A., Gamer, A.O., van Ravenzwaay, B. & Landsiedel, R. (2010). Experience with the HET-CAM method in the routine testing of a broad variety of chemicals and formulations. *ATLA* **38**, 39–52.
12. UN (2013). *Globally Harmonized System of Classification and Labelling of Chemicals (GHS) Fifth Revised Edition*. Geneva, Switzerland: United Nations Economic Commission for Europe.
13. EPA (1996). *Label Review Manual 2nd Edition*, 168pp. Washington, DC, USA: Environmental Protection Agency.
14. Anon. (1992). *Portaria Nº 03/MS/SNVS, de 16 de Janeiro de 1992*. Brasília, DF, Brazil: Imprensa Nacional.
15. OECD (2008). *OECD Guidance for Country Data Review Reports on Plant Protection Products and their Active Substances* (*OECD Monograph Guidance)*, 32pp. Paris, France: Organisation for Economic Co-operation and Development.
16. Draize, J.H., Woodard, G. & Calvery, H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology & Experimental Therapeutics* **82**, 377–390.
17. Gautheron, P., Dukic, M., Alix, D. & Sina, J.F. (1992). Bovine corneal opacity and permeability test: An *in vitro* assay of ocular irritancy. *Fundamental & Applied Toxicology* **18**, 442–449.
18. OECD (2011). *Series on Testing and Assessment No. 160: Guidance Document on the Bovine Corneal Opacity and Permeability (BCOP) and Isolated Chicken Eye (ICE) Test Methods: Collection of Tissues for Histological Evaluation and Collection of Data on Non-Severe Irritants*, 63pp. Paris, France: Organisation for Economic Co-operation and Development.
19. Jester, J.V., Petroll, W.M., Bean, J., Parker, R.D., Carr, G.J., Cavanagh, H.D. & Maurer, J.K. (1998). Area and depth of surfactant-induced corneal injury predicts extent of subsequent ocular responses. *Investigative Ophthalmology & Visual Science* **39**, 2610–2625.
20. Jester, J.V. (2006). Extent of corneal injury as a biomarker for hazard assessment and the development of alternative models to the Draize rabbit eye test. *Cutaneous & Ocular Toxicology* **25**, 41–54.
21. Maurer, J.K., Parker, R.D. & Jester, J.V. (2002). Extent of initial corneal injury as the mechanistic basis for ocular irritation: Key findings and recommendations for the development of alternative assays. *Regulatory Toxicology & Pharmacology* **36**, 106–117.
22. Kaluzhny, Y., Kandárová, H., Hayden, P., Kubilus, J., d'Argembeau-Thornton, L. & Klausner, M. (2011). Development of the EpiOcular™ eye irritation test for hazard identification and labelling of eye irritating chemicals in response to the requirements of the EU cosmetics directive and REACH legislation. *ATLA* **39**, 339–364.
23. Adriaens, E., Barroso, J., Eskes, C., Hoffmann, S., McNamee, P., Alepee, N., Bessou-Touya, S., De Smedt, A., De Wever, B., Pfannenbecker, U., Tailhardat, M. & Zuang, V. (2014). Retrospective analysis of the Draize test for serious eye damage/eye irritation: Importance of understanding the *in vivo* endpoints under UN GHS/EU CLP for the development and evaluation of *in vitro* test methods. *Archives of Toxicology* **88**, 701–723.
24. Scott, L., Eskes, C., Hoffmann, S., Adriaens, E., Alepee, N., Bufo, M., Clothier, R., Facchini, D., Faller, C., Guest, R., Harbell, J., Hartung, T., Kamp, H., Varlet, B.L., Meloni, M., McNamee, P., Osborne, R., Pape, W., Pfannenbecker, U., Prinsen, M., Seaman, C., Spielmann, H., Stokes, W., Trouba, K., Berghe, C.V., Goethem, F.V., Vassallo, M., Vinardell, P. & Zuang, V. (2010). A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-Up and Top-Down approaches. *Toxicology in Vitro* **24**, 1–9.
25. EPA (2013). *Use of an Alternate Testing Framwork for Classification of Eye Irritation Potential of EPA Pesticide Products*, 39pp. Washington, DC, USA: Environmental Protection Agency.