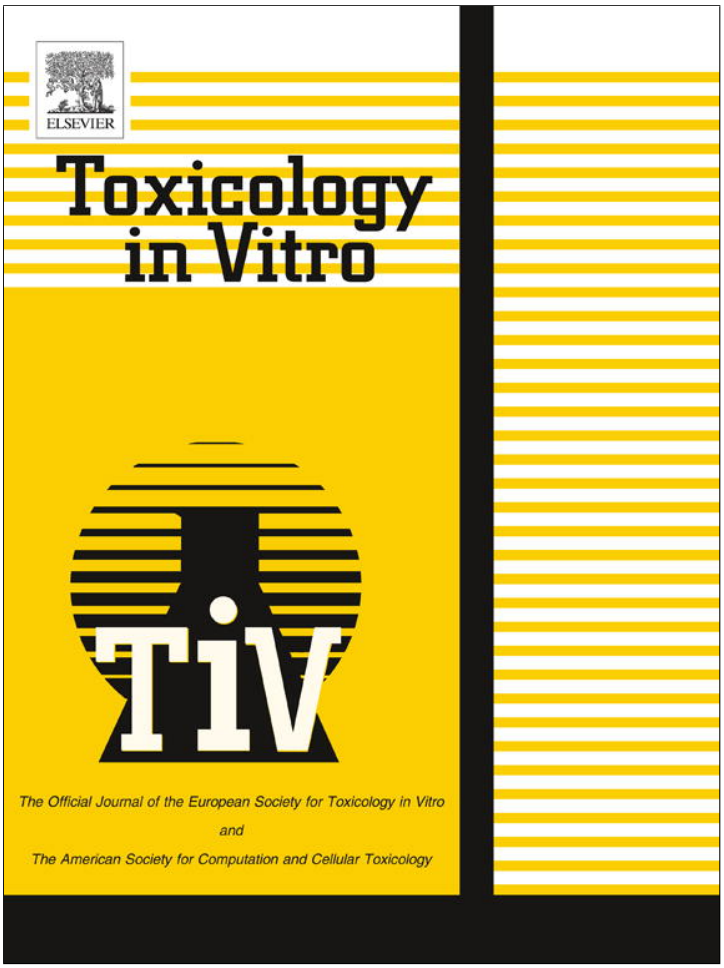

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Improvement of the Bovine Corneal Opacity and Permeability (BCOP) assay as an *in vitro* alternative to the Draize rabbit eye irritation test

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ABSTRACT

Measurement of ocular irritancy is a necessary step in the safety evaluation of both industrial and consumer products. Assessment of the acute eye irritation potential is therefore part of the international regulatory requirements for testing of chemicals.

The Bovine Corneal Opacity and Permeability (BCOP) assay is generally accepted as a valid *in vitro* alternative method to the Draize eye irritation test to detect corrosive and severe eye irritants (category 1), but has not proven sensitive enough to discriminate accurately moderate (category 2A/2B) to mild and non-irritating compounds. In the currently accepted BCOP assay, opacity is determined by the amount of light transmission through the cornea, and permeability is determined by the amount of sodium fluorescein dye that passes through all corneal cell layers. Both measurements are used to assign an *In Vitro* Irritancy Score (IVIS) for prediction of the *in vivo* ocular irritation potential of a test substance. Nowadays, opacity is measured by an OP-KIT opacitometer providing a center-weighted reading of light transmission by measuring changes in voltage when the transmission of white light passes through the cornea alters. As a consequence, this may underestimate opacity that develops as spots or heterogeneous opaque areas on the periphery of an isolated cornea.

A prototype of a laser light-based opacitometer (PLLBO) allowing better measurement of opacities was developed by Van Goethem et al. (2010). This new device showed improved sensitivity to detect subtle changes in corneal transparency. Furthermore, the new opacitometer allowed the analysis of the complete corneal surface and was able to detect more efficiently opaque spots located along the sides of the excised corneas.

A further improved prototype of the PLLBO was constructed in combination with a camera and a speckle noise reducer. Treatment conditions of the corneas in the cornea holders were optimized in order to mimic more the real *in vivo* situation. A set of test compounds with irritancy potencies especially in the mild and moderate range was tested. The improved LLBO showed some promising features which potentially could improve the usefulness of the BCOP test. Adaptation of cornea holders showed to be of limited value and only restricted to concentrations up to 15% which mimics more test conditions in industry.

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1. Introduction

The human eye is a complex organ comprised of various different tissues and cell types. The cornea, constituting the outer barrier of the eye, and the conjunctivae are the tissues which are most often affected after exposure of the eye to ocular irritants (Hackett and McDonald, 1991). Eye irritation is the result of changes in the eye following the application of a test chemical to the anterior surface of the eye and which are fully reversible within 21 days of application. Measurement of ocular irritancy is a necessary step in the safety evaluation of both industrial and consumer products.

Irritation testing using laboratory animals has largely remained unchanged for many years. The Draize eye irritation test (Draize et al., 1944) became a governmentally endorsed methodology and is described in OECD testing guideline (TG) 405 (OECD, 2002). Advances in ocular toxicology are challenging the validity, precision, and relevance of the Draize eye irritation test (Wilhelmus, 2001). Significant levels of variability were observed since the test is based on a subjective scoring procedure (Weil and Scala, 1971). Besides the fact that the test causes considerable discomfort and pain to animals, it is also recognized that the response in the

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rabbit is not always predictive of that found in humans (Curren and Harbell, 2002; Freeberg et al., 1986; Griffith et al., 1980). Taken together, both ethical and scientific reasons stimulated the development and validation of several alternative *in vitro* methods to assess eye irritation (Balls et al., 1999; Worth and Balls, 2002).

The status of alternative methods for eye irritation was described by Eskes et al. (2005) (Eskes et al., 2005) and although some of the assays showed considerable promise as screens for ocular irritancy, the outcome of many validation studies concluded that no single test was capable of replacing the range of injuries measured in the Draize rabbit eye test. The main reason for this is the difficulty of comparing *in vitro* test results with historical animal data which are often of insufficient quality for validation purposes. Further, one should realize that the *in vitro* tests only partially models the complex *in vivo* eye irritation response. However, a number of tests, including the BCOP (Bovine Corneal Opacity and Permeability), HET-CAM (Hen's Egg Test-ChorioAllantoic Membrane), IRE (Isolated Rabbit Eye), and ICE (Isolated Chicken Eye) tests, are accepted by European Union (EU) national regulatory authorities, on a case-by-case basis, for the identification of corrosive and severe eye irritants (EU, 2004).

The BCOP assay is an *in vitro* alternative which is routinely used in several industrial and contract testing laboratories in the context of workplace safety and product safety applications and is described in OECD TG 437 (OECD, 2009). However, the BCOP assay seems to be not sensitive enough to discriminate among moderate and mildly irritating materials when applying the standard protocol (Cooper et al., 2001; Eskes et al., 2005; Jones et al., 2001).

The assay is based on the methods described by Muir (1984, 1985, 1987) and Tchao (1988) and was developed by Gautheron et al. (1992) for the prediction of the irritation potential of process intermediates and compounds in development. In general, the BCOP assay can test a wide range of physical forms and solubility characteristics. For example, Vanparys et al. (1993) showed that when fifty pharmaceutical and commercially available substances were evaluated representing both liquids (miscible and immiscible) and solids (soluble and insoluble), a concordance of 96% was obtained when irritants were discriminated from non-irritants.

Up to now opacity readings are performed by using the OP-KIT opacitometer, which is a white (polychromatic) light, dual-beam opacitometer. This type of opacitometer provides a center-weighted reading of light transmission through the cornea. It was found that with the OP-KIT opacitometer different values on opacity were obtained (specially with alcohols and solids) depending where the spot was located, leading to misclassification of compounds for their eye irritating potential (Van Goethem et al., 2010). Another observation was that the OP-KIT opacitometer is not sensitive enough to differentiate between moderate (category 2A/2B), mild, and non-irritating compounds (narrow scale for ranking compounds). Furthermore, the OP-KIT device is difficult to calibrate and is not stable over time.

Recognizing the limitations of the conventional OP-KIT opacitometer, a prototype laser light-based opacitometer (PLLBO) was developed that uses an adjustable laser beam in combination with a calibrated photocell instead of visible light. This new opacitometer was designed to provide a more even distribution of light across the corneal surface which resulted in an improved method of opacity assessment when optical linearity and local effects (opaque spot induction) were analyzed (Van Goethem et al., 2010). Additional studies are required to determine if such instruments provide an improvement over the conventional opacitometer. This proof of concept should be considered as a first step towards a technical optimization in which further possible advantages of laser light technology should be investigated. For that reason, an improved PLLBO was constructed by using the laser light-based opacitometer (LLBO) in combination with a camera and a speckle noise reducer and by modifying the cornea holders so that test compounds and formulations can be diluted whether or not in function of time as happens *in vivo* by excessive tearing in the eye of man at exposure which attempt to wash out irritants that may have come into contact with the eye.

A set of compounds with irritancy potencies especially in the mild and moderate range were tested and compared with the results of the original PLLBO when possible. The improved LLBO showed promising features which potentially could improve the usefulness and applicability domain of the BCOP test.

2. Materials and methods

2.1. Test compounds

The eye irritating potential of nine reference chemicals (Table 1) selected from the recommended substances list for demonstrating technical proficiency with BCOP of OECD TG 437 (OECD, 2009) was assessed. The selected reference chemicals, for which high quality *in vivo* rabbit eye test data (ECETOC, 1998; Gautheron et al., 1994) and high quality *in vitro* BCOP data (Balls et al., 1999; Gautheron et al., 1994) exist, covered the range of irritant categories from non to severe (category 1) and represent different chemical classes.

The eye irritating potential of another 20 compounds (Table 2) selected from literature was assessed to evaluate further the performance of the new LLBO (Balls et al., 1999; Gautheron et al., 1994). The selection of test compounds focused on the non- to moderate (category 2A/2B) irritant categories and represent different chemical classes.

To determine the suitability of the adapted cornea holders, concentration series (15–100%) of ethyl acetoacetate (Table 2) and N,N-dimethylformamide (DMF, CAS: 68-12-2) were used. The latter is also used as positive control for liquid test items in the BCOP assay.

Also cosmetic formulations and simple formulations with different irritancy potencies were tested. Since it was not possible to obtain formulations from industry, it was decided to test three commercial kids shampoos labeled as 'does not sting the eyes'. The shampoos were tested as the actual formulation as they are on the market. Simple formulations were made of irritating compounds diluted in artificial tear fluid (Hyabak). A concentration series was tested for one severely irritating (pyridine), one moderately irritating (γ-butyrolactone), and one mildly irritating (1,2,4-trimethylbenzene) compound. All three compounds were previously tested as pure products (100%) by others.

2.2. BCOP assay

The BCOP assay was performed according to OECD TG 437 (OECD, 2009). A brief overview can be found in the paper of Van Goethem et al. (2010).

2.3. Construction of an improved laser light-based opacitometer (LLBO)

2.3.1. Conventional opacitometer (OP-KIT)

Up to now, opacity readings are performed by using the OP-KIT opacitometer (MC2, Clermont Ferrand Cedex, France), which is based on the use of a white (polychromatic) light, dual-beam opacitometer. This type of opacitometer provides a center-weighted reading of light transmission through the cornea. More detailed technical information on this device can be found in the paper of Van Goethem et al. (2010).

Table 1
Reference chemicals selected from OECD test guideline 437.

Chemical	CAS	Chemical class[a]	Physical form
Benzalkonium chloride (5%)	8001-54-5	Onium compound	Liquid
Dibenzoyl-L-tartaric acid	2743-38-6	Carboxylic acid, ester	Solid
Imidazole	288-32-4	Herterocyclic	Solid
Trichloroacetic acid (30%)	76-03-9	Carboxylic acid	Liquid
2,6-Dichlorobenzoyl chloride	4659-45-4	Acyl halide	Liquid
Ethyl-2-methylacetoacetate	609-14-3	Ketone, ester	Liquid
Ammonium nitrate	6484-52-2	Inorganic salt	Solid
Glycerol	56-81-5	Alcohol	Liquid
n-Hexane	110-54-3	Hydrocarbon (acyclic)	Liquid

[a] Chemical classes were assigned to each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system.

Table 2
Twenty chemicals selected from literature [9, 23].

In vitro irritation class	Chemical	CAS	Chemical class	Physical form
Non	L-Aspartic acid	70-47-3	Amino acids	Solid
	Polyethylene glycol 400	25322-68-3	Alcohol, polyether	Liquid
	2-Mercaptopyrimidine	1450-85-7	Acyl halide	Solid
	EDTA, dipotassium salt	25102-12-9	Amine, carboxylic acid (salt)	Solid
	Anthracene	120-12-7	Polycyclic	Solid
Mild	Cetylpyridinium bromide	140-72-7	Heterocyclic, onium compound	Liquid
	Methyl cyanoacetate	105-34-0	Ester, nitrile compound	Liquid
	Potassium cyanate	590-28-3	Inorganic salt	Solid
	Tetraaminopyrimidine sulfate	5392-28-9	Amine, heterocyclic, inorganic salt	Solid
	Propyl-4-hydroxybenzoate	94-13-3	Carboxylic acid, phenol	Solid
	1-Nitropropane	108-03-2	Hydrocarbon (acyclic), nitro compound	Liquid
	3-Glycidyloxypropyltrimethoxysilane	2530-83-8	Organosilicon compound	Liquid
	Dimethyl sulfoxide	67-68-5	Organic sulfur compound	Liquid
	Methylisobutylketone	108-10-1	Ketone	Liquid
	Petroleum ether	8032-32-4	Hydrocarbon (acyclic)	Liquid
Moderate	Parafluoraniline	371-40-4	Amine/amidine	Liquid
	Ethyl acetoacetate	141-97-9	Carboxylic acid, ketone	Liquid
	Hexadecyltrimethylammonium bromide	57-09-0	Organic salt, onium compound	Liquid
	Butyl acetate	123-86-4	Ester	Liquid
	Sodium lauryl sulfate (3%)	151-21-3	Carboxylic acid (salt)	Liquid

2.3.2. Prototype laser light-based opacitometer (PLLBO)

A new opacitometer (PLLBO) was designed at Janssen Pharmaceutica N.V. with laser light as light source instead of visible light. The characteristics of visible versus laser light-based opacitometers are shown in Table 3. A helium–neon (HeNe) laser (Melles Griot BV, The Netherlands) was used. Its operation wavelength is 632.8 nm producing a 5.0 mW coherent, random polarized monochromatic light beam (0.8 mm) in the red portion of the visible spectrum. A photometer (Minolta certified lux meter) was used with following specifications: digital read-out, 2% accuracy, range between 0.01 and 99,900 lux, silicon photocell. The unit of measurement is lux, the International System of Units of illuminance. Start values were routinely set at 1210 lux when an empty cornea holder was placed in the PLLBO, since intensity can be regulated by an adjustable neutral density filter (NDF). The technical optimization and optical characteristics of this device can be found in the paper of Van Goethem et al. (2010).

Table 3
Characteristics of OP-KIT and laser-light based opacitometers.

Visible light-based opacitometer (OP-KIT)	Laser light-based opacitometers
Polychromatic	Monochromatic
Non-linear	Linear
Two light sources of different life time	One light source
Only a part of the cornea is analyzed	The whole cornea is analyzed
–	The width of the light beam can be adjusted

2.3.3. Technical specifications improved LLBO

A copy of the PLLBO of Janssen Pharmaceutica N.V. was constructed with further technical improvement of the equipment for opacity measurements regarding type and specifications. A helium–neon (HeNe) green laser (Melles Griot BV, The Netherlands) was selected instead of a red laser to enlarge the sensitivity. Its operation wave-length is 543.5 nm producing a 10.0 mW coherent, random polarized monochromatic light beam (0.81 mm) in the green portion of the visible spectrum. Furthermore, the LLBO was constructed in combination with a camera (12 bit TXG50 5 Megapixel from Baumer) and a speckle noise reducer. Also a Bi-Telecentriclens for 2/3″ chips was used to prevent distortion on the 2D picture. Both the Minolta lux measurement device and digital camera were constructed on a linear slide to obtain a more flexible setting. The advantages of including a camera on top of a calibrated photocell allowing the possibility to capture images to obtain extra information on possible injury (data not shown) and the possibility to perform densitometry measurements (future perspectives). Laser light is coherent with the induction of speckle noise, which disturbs image analysis with a camera. A speckle noise reducer is necessary to reduce the noise and to obtain a perfect sharp image.

Peira Scientific Instruments (Beerse, Belgium) constructed the LLBO as detailed in Fig. 1A. Intensity of laser light can be regulated by an adjustable NDF. Based on preliminary experiments start values were routinely set at 2000 lux with placement of an empty cornea holder.

2.3.4. Heterogeneous opacity measurement

The correctness of opacity measurement when using a center-weighted reading was evaluated by heterogeneous opacity induction of the cornea using three different approaches in analogy as described in Van Goethem et al. (2010). A first approach was performed by blocking the maximum amount of light with artificial black spots of different diameters. The black spots were placed on different locations on a transparent filter and held in front of a cornea when opacity values were recorded. Secondly, local opacity was induced by a 10 min exposure of the corneas to DMF-saturated absorbent paper (6 mm diameter) at the center or periphery. Opacity was assessed with the OP-KIT and LLBO after 2 h recovery. Thirdly, the effect of inaccurate cornea treatment was reproduced by heterogeneous exposure of corneas to methanol. In this experiment three corneas were completely treated and three corneas were only treated at the periphery with methanol. Results were compared with those obtained using the PLLBO.

2.3.5. Performance assessment of LLBO

To evaluate the performance of the improved LLBO and to built a new prediction model to classify the test compounds, the eye irritating potential of 29 reference chemicals (Tables 1 and 2) was assessed. Three corneas per concentration were treated for 10 min (liquids) or 4 h (solids) and opacity was assessed with the OP-KIT and LLBO after 2 h recovery (liquids) or immediately (solids). At least two independent experiments were performed for each chemical. *In Vitro Scores* and classifications were calculated according to the proposed prediction models (Table 4). For each experiment, the average IVIS of three treated corneas with corresponding standard deviations (SD) are presented. To compare the IVIS results of the LLBO with those of the OP-KIT, only absolute lux values were considered. It is important to mention that only one direction, namely the increase of opacity in time, was taken into account. This means that when corneas show an improvement in light permeability (negative value) in time, the lux values were set to zero.

2.3.6. Prediction models

The *In Vitro* Irritation Score (IVIS) was calculated by combining both opacity and permeability results and three different prediction models are used for the three devices (OP-KIT, LLBO, and PLLBO) (Table 4). For the LLBO, opacity was defined as the amount of lux divided by 7 (lux/7) in order to transform the obtained illuminance values in the range of the standard OP-KIT prediction model.

2.4. Adaptation of cornea holders

The adapted cornea holder was designed by Peira Scientific Instruments (Fig. 1B). There are two extra small holes foreseen in the cornea holder. If the cornea holder is fielded horizontally with the two small round holes facing to the top then the flush tool can be installed. In this flush tool are two small holes (sideward positioned). If the flush tool is mounted in the two small holes of the cornea holder then the two small holes of the flush tool are in the center of the mounted cornea. Herewith is assured when the flushing starts that there is an immediate impact on the cornea and thus this is an attempt to mimic tears.

In the BCOP assay, opacity and permeability measurements are used to assign an IVIS for prediction of the *in vivo* ocular irritation potential of a test substance. For the experiments with the adapted cornea holder, only opacity was determined assumed that this

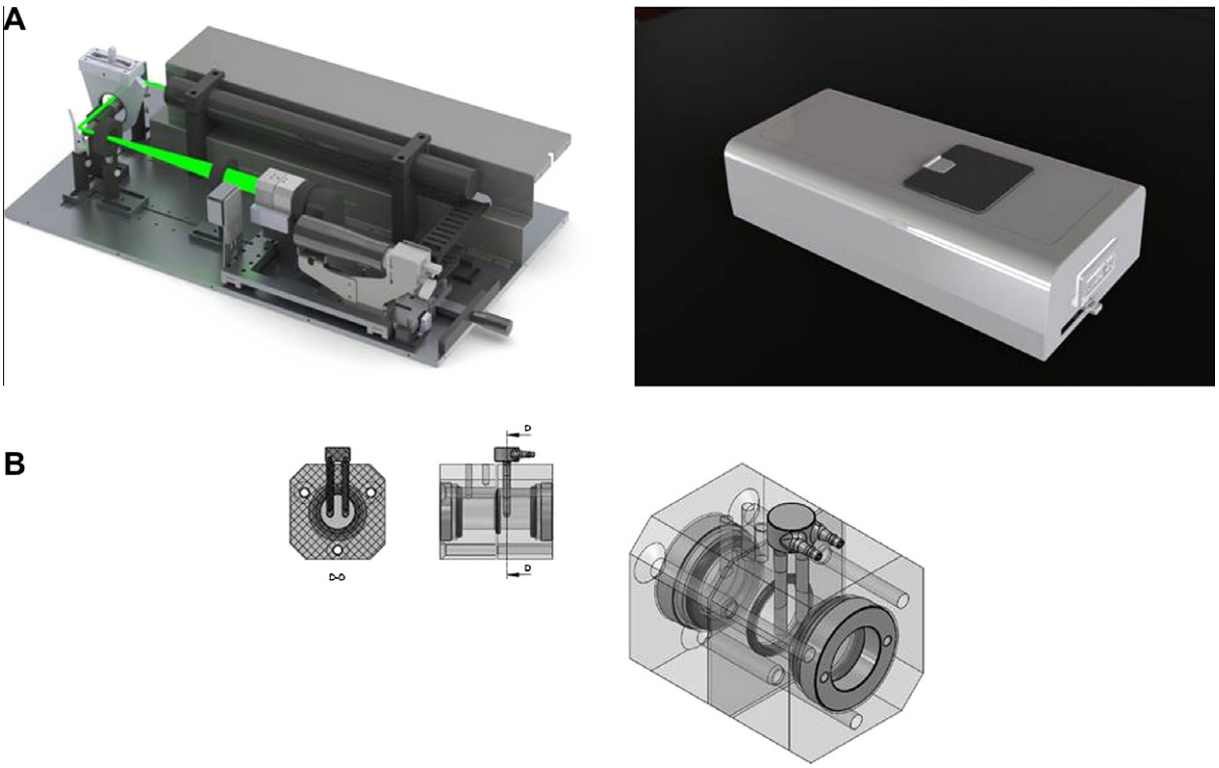

Fig. 1. Design laser light-based opacitometer (A), and adapted cornea holder (B).

Table 4
Calculations and prediction models opacity was measured with the conventional OP-KIT, the laser light-based opacitometer (LLBO), and the prototype laser light-based opacitometer (PLLBO). *In Vitro* Irritation Scores (IVIS) were calculated.

IVIS OP-KIT	IVIS LLBO	IVIS PLLBO	*In vitro* classification
⩽3	⩽20	⩽10	Non-irritant
3.1–25	20.1–75	10.1–50	Mild irritant
25.1–55	75.1–125	50.1–100	Moderate irritant
>55	>125	>100	Severe irritant

IVIS OP-KIT: Opacity + 15 times OD_{490}.
IVIS LLBO: Laser light-based opacitometer IVIS = (lux/7) + 15 times OD_{490}.
IVIS PLLBO: Prototype laser light-based opacitometer IVIS = (lux/6) + 15 times OD_{490}.

endpoint is the first affected by dilution of the test compound. To determine the suitability of the adapted cornea holders, concentration series (15–100%) of compounds were used as detailed in Section 2.1. For practical reasons, 0.9% sodium chloride was used for dilution of the compounds. Here, concentrations up to 15% were tested for practical considerations (volume restriction of cornea holder of 5 ml). Three corneas were treated with a volume of 750 µl test compound. Opacity was determined at baseline and after 2 h post-treatment using the OP-KIT device and LLBO. Other sets of three corneas were used to analyze the effect of dilution of the test items on average opacity readings. After 1 out of 10 min exposure of the corneas to the test compound, the latter was diluted in the cornea holder by adding different volumes of 0.9% sodium chloride to obtain a concentration series from 100% to 15%. In addition, a dilution (15–66.7%) with 0.9% sodium chloride was added to a set of three corneas already after 5, 10, 20, or 30 s to have an idea if timing of dilution has an impact on opacity readings. It was also checked if removing of the substance after injection may have an impact on opacity readings for a moderate eye irritant. After substracting the baseline opacity from the post-treatment opacity reading for each individual cornea, the average opacity reading and corresponding SD was calculated for each set of three corneas. A Student's *t*-Test (two-tailed distribution, unequal variance) was performed to determine if dilution of the test compound is significantly different ($p < 0.05$) compared to the initial concentration of the compound.

2.5. Testing an expanded set of formulations

Commercially available baby shampoos labeled as 'does not sting the eyes' and simple formulations of pyridine, γ-butyrolactone, and 1,2,4-trimethylbenzene with different irritancy potencies were tested using the LLBO and OP-KIT. The shampoos are tested as the actual formulation as it is on the market. Four concentrations (25–50–75–100%) were tested of the simple formulations to compare the irritancy ranking. *In Vitro Scores* and classifications were calculated according to the proposed prediction models (Table 4). For each experiment, the average IVIS of three treated corneas with corresponding SD are presented.

3. Results

3.1. Construction of an improved laser light-based opacitometer (LLBO)

Using the improved LLBO, we observed that laser light was subject to some minor variation (±1.5%), but this fluctuation is negligible in the BCOP assay. In an experimental setup, each of the three corneas are treated with test substance. This means that we also have to deal with biological variation between the three corneas measured. To avoid interference of the light source, it was decided to switch on the laser light-based device 30 min before starting the experiments to improve stability of the laser light.

3.1.1. Heterogeneous opacity readings

In order to evaluate if heterogeneous opacity induction can result in the underestimation of the irritating potential of test substances, peripheral effects on the cornea were mimicked by three different approaches.

First, artificial black spots with different diameters were placed on different locations of a transparent filter and opacity measurements were performed both with the OP-KIT and LLBO. Results were also compared with those using the PLLBO. When opacity values of peripheral spots were compared with opacity values of spots (same size) in the center (Table 5), both opacitometers showed unbalanced readings (although the effect is more pronounced with the OP-KIT), especially for smaller spots (3 and 6 mm). To aid in data interpretation, the opacity values of each peripheral spot was calculated as a percentage of the respective central reading, which was set at 100%. Differences were observed between the OP-KIT and the LLBO for these weighted data points. A spot of 3 mm was almost totally underestimated with the OP-KIT, when located at the periphery since opacity values ranged between 0% and 2.6%. Also with the larger spots, a marked lower opacity reading was noted when the spots are located at the periphery of the cornea.

Unbalanced readings also occurred with the LLBO and PLLBO when a spot was located at the periphery. Nevertheless, when located at the periphery, a spot of 3 mm was less underestimated with the LLBO. Peripheral values ranged from 64% to 85% in comparison with 28.2% to 49.3% for the PLLBO. The same conclusion can be made for the larger peripheral spots (6, 9, and 12 mm). With the LLBO, a spot of 6, 9, and 12 mm had a peripheral range of

Table 5
Induced opacity values when artificial black spots were placed on the periphery and center of a transparent filter, measured with the OP-KIT and laser light-based opacitometer (LLBO). Results were compared with the results obtained using the prototype laser light-based opacitometer (PLLBO). Values in bold are the measured values, while those in normal format are the% compared to the concurrent middle spot measurement set at 100%. Legend: AU = arbitrary units.

Diameter (mm)		Opacity values				
		Left	Right	Top	Bottom	Center
						
OP-KIT	3	**0.3**	**0.0**	**−0.1**	**0.2**	**11.6**
(AU)		2.6	0.0	0.0	1.7	100.0
	6	**2.4**	**4.0**	**2.7**	**3.4**	**27.5**
		8.7	14.5	9.8	12.4	100.0
	9	**22.7**	**17.1**	**24.1**	**23.2**	**65.4**
		34.7	26.1	36.9	35.5	100.0
	12	**67.9**	**78.3**	**76.0**	**109.9**	**189.1**
		35.9	41.4	40.3	58.1	100.0
LLBO (lux)	3	**85.0**	**64.0**	**76.0**	**69.0**	**100.0**
		85.0	64.0	76.0	69.0	100.0
	6	**383.0**	**145.0**	**244.0**	**207.0**	**414.0**
		92.5	35.0	58.9	50.0	100.0
	9	**680.0**	**269.0**	**635.0**	**385.0**	**791.0**
		86.0	34.0	80.3	48.7	100.0
	12	**1120.0**	**780.0**	**996.0**	**856.0**	**1007.0**
		111.2	77.5	98.9	85.0	100.0
PLLBO	3	**40.0**	**50.0**	**70.0**	**40.0**	**142.0**
(lux)		28.2	35.2	49.3	28.2	100.0
	6	**110.0**	**110.0**	**168.0**	**140.0**	**336.0**
		32.7	32.7	50.0	41.7	100.0
	9	**345.0**	**416.0**	**488.0**	**354.0**	**614.0**
		56.2	67.8	79.5	57.7	100.0
	12	**798.0**	**817.0**	**826.0**	**736.0**	**915.0**
		87.2	89.3	90.3	80.4	100.0

respectively 35.0% to 92.5%, 34% to 86%, and 77.5% to 111.2%. With the PLLBO the values of a spot of 6, 9, and 12 mm ranged from 32.7% to 50.0%, 56.2% to 79.5%, and 80.4% to 90.3%, respectively.

Secondly, local opacity was induced by a 10 min exposure of the corneas to DMF-saturated absorbent paper at the center or periphery. Opacity was assessed with the OP-KIT and the LLBO after 2 h recovery and results were compared with those of the PLLBO. Unbalanced opacity values were seen depending on the location of the spot (Table 6). In analogy with the previous calculations, peripheral values were 19.9% (OP-KIT) and 41.7% (LLBO). This confirms the improved balanced output of the LLBO. Permeability was less dependent on the location of the spot and was used to calculate the IVIS. A shift in irritation category (form moderate (category 2A/2B) to mild) was observed for the OP-KIT when comparing central induced IVIS with peripheral induced IVIS. This shift was not observed for the LLBO thus supporting the technical improvements of this new opacitometer in comparison with the OP-KIT. In the study of Van Goethem, the peripheral values of the corneas after a 10 min treatment with DMF-saturated absorbent paper were comparable to the results of the LLBO. Based on a peripheral value of 20.0% for the OP-KIT (data not shown) and 60.2% for the PLLBO they also confirmed the improved balanced output of the PLLBO. However, based on the specific prediction models, corneal effects were classified in different categories depending on the location of the spot. A shift occurred from moderate (category 2A/2B) to mild when peripheral opacity was induced.

Finally, inaccurate cornea treatment was reproduced by partially exposing the cornea to methanol for 10 min and comparing these results with the IVIS induced after full cornea treatment (Table 7). The results demonstrated the center-weighted reading technique of the OP-KIT, as the OP-KIT determined a mean opacity value of 11.0 for the partial treatment and 51.3 when the complete cornea was exposed. In analogy with the previous calculations the weight of the partial treatment was 21.4%. When comparing the OP-KIT with the LLBO, the LLBO underestimated less the peripheral induced opacity as the weight of the partial treatment was 61.5%. As expected, the permeability decreased when only the peripheral zone of the cornea was damaged, an IVIS classification shift from severe (category 1) to mild was observed for the OP-KIT, and from severe (category 1) to moderate (category 2A/2B) for the LLBO. The same classification shifts were observed for the PLLBO. However, a slightly lower weight for the partial treatment was observed for the PLLBO (54.4%).

3.1.2. Performance assessment

The eye irritating potential of nine reference chemicals (Table 1) was assessed to evaluate the performance of the improved LLBO. Data on opacity was generated with the LLBO and in parallel also data was generated with the OP-KIT opacitometer. Both sets of data were compared with each other and with existing *in vivo* (OECD TG 405) and *in vitro* (OECD TG 437 and 438) classifications (Table 8).

Following *in vivo* classification, 2 chemicals are not labeled, 3 chemicals are classified in category 2A/2B (A = irritating to the eye, which is reversible in 21 days and B = mildly irritating to the eye, which is reversible in 7 days), and 4 chemicals are classified in category 1 (irreversible effects on the eye). The *in vitro* classification identified 5 non-corrosive/non-severe eye irritants and 4 corrosive/severe (category 1) eye irritants according to the OECD TGs. The *in vitro* BCOP IVIS data obtained with the OP-KIT device identified 3 non-irritants, 2 mild irritants, no moderate (category 2A/2B) irritants, and 4 severe (category 1) irritants. The *in vitro* BCOP IVIS data obtained with the LLBO identified 2 non-irritants, 2 mild irritants, 1 moderate irritant, and 4 severe irritants. According to OECD TG 437, all IVIS data generated by both devices are classified correctly as (non)-corrosive/(non)-severe eye irritants compared to the *in vitro* classification. Three independent experiments were performed for the chemical 2,6-dichlorobenzoyl chloride and one out of three experiments gave an LLBO IVIS of borderline non-irritant, close to mild-irritating, whereas in the standard OP-KIT all three independent experiments classified this chemical as a non-eye irritant. Ammonium nitrate, also tested in triplicate, was classified as mild and moderately irritating to the eye, respectively in the OP-KIT and LLBO.

Furthermore, the eye irritating potential of another 20 compounds (Table 2) in the non- to moderate (category 2A/2B)

Table 6
Corneal opacity and permeability induction after 10 min treatment at the center or periphery with a N,N-dimethylformamide (DMF)-saturated filter paper (6 mm diameter). opacity was measured with the OP-KIT and laser light-based opacitometer (LLBO) and results were compared with data of the prototype laser light-based opacitometer (PLLBO). *In vitro* Irritation Scores (IVIS) were calculated according to Table 4.

Device	DMF treatment	Opacity	Permeability	IVIS	Irritation class
OP-KIT	Center	27.6 ± 15.4	0.298 ± 0.323	32.1 ± 18.1	Moderate
	Periphery	5.5 ± 1.7 (19.9%)	0.272 ± 0.424	9.5 ± 7.9	Mild
LLBO	Center	346.7 ± 137.9	0.298 ± 0.323	56.2 ± 31.1	Mild
	Periphery	144.7 ± 37.0 (41.7%)	0.272 ± 0.424	30.3 ± 8.2	Mild
PLLBO	Center	298.6 ± 120.7	0.175 ± 0.072	52.4 ± 19.0	Moderate
	Periphery	179.6 ± 24.7 (60.2%)	0.145 ± 0.116	32.1 ± 5.6	Mild

Table 7
Corneal opacity and permeability induction after complete and partial 10 min treatment with methanol. Corneal opacity and permeability were measured with the OP-KIT and the laser light-based opacitometer (LLBO) and results were compared with data of the prototype laser light-based opacitometer (PLLBO). *In vitro* Irritation Scores (IVIS) were calculated according to Table 4.

Device	Methanol treatment	Opacity	Permeability	IVIS	Irritation class
OP-KIT	Complete cornea	51.3 ± 21.3	1.466 ± 0.927	73.0 ± 13.2	Severe
	Periphery	11.0 ± 3.3 (21.4%)	0.185 ± 0.069	13.8 ± 2.6	Mild
LLBO	Complete cornea	1279.2 ± 143.4	1.466 ± 0.927	202.8 ± 18.1	Severe
	Periphery	786.3 ± 96.4 (61.5%)	0.185 ± 0.069	111.3 ± 14.2	Moderate
PLLBO	Complete cornea	860.3 ± 33.3	2.585 ± 0.228	182.2 ± 7.6	Severe
	Periphery	468.3 ± 99.5 (54.4%)	0.462 ± 0.115	85.0 ± 17.6	Moderate

Table 8
In Vitro Irritancy Scores of 9 reference chemicals (Table 1).

Chemical	Study number	IVIS OP-KIT Average	IVIS OP-KIT SD	IVIS LLBO Average	IVIS LLBO SD	*In vitro* classification	*In vivo* classification
Benzalkonium chloride (5%)	1	119.4	4.5	257.8	7.9	Corrosive/ Severe eye irritant	Category 1
	2	126.9	12.6	257.9	11.8		
Dibenzoyl-L-tartaric acid	1	128.7	15.2	234.4	3.6	Corrosive/ Severe eye irritant	Category 1
	2	142	30.5	223.9	8.0		
Imidazole	1	89.5	9.1	235.7	20.0	Corrosive/ Severe eye irritant	Category 1
	2	84.9	2.1	204.4	3.3		
Trichloroacetic acid (30%)	1	211.8	41.8	275.5	2.5	Corrosive/ Severe eye irritant	Category 1
	2	181.1	5.1	275.4	15.2		
2,6-Dichlorobenzoyl chloride	1	2.5	1.9	37.2	4.1	Non-corrosive/ non-severe eye irritant	Category 2A
	2	1.5	0.7	21.2	2.9		
	3	2.3	2.1	19.4	9.6		
Ethyl-2-methylacetoacetate	1	9.3	0.5	52.1	8.9	Non-corrosive/ non-severe eye irritant	Category 2B
	2	6.9	2.1	63.2	1.4		
Ammonium nitrate	1	10.2	1.3	90.9	7.8	Non-corrosive/ non-severe eye irritant	Category 2A
	2	8.3	0.7	104.2	3.8		
	3	11.2	4.1	79.9	7.1		
Glycerol	1	0.1	0.18	-0.8	0.5	Non-corrosive/ non-severe eye irritant	Not Labeled
	2	1.7	0.8	0.0	0.0		
n-Hexane	1	2.3	1.1	18.9	8.2	Non-corrosive/ non-severe eye irritant	Not Labeled
	2	2.5	0.6	12.0	7.4		
	3	0.6	0.3	0.6	1.1		

In Vitro Irritancy Scores (IVIS) classifications after measurement of opacity with the standard OP-KIT and laser light-based opacitometer (IVIS LLBO), calculated according to Table 4, were compared with high quality *in vivo* data (based on results from the *in vivo* rabbit eye test (OECD TG 405) and using Global Harmonized System (GHS) classification) and *in vitro* data (based on results in Bovine Corneal Opacity test (OECD TG 437) and Isolated Chicken Eye test (OECD TG 438)). At least two independent experiments were performed for each chemical for the calculations of IVIS OP-KIT and LLBO and for each experiment, the average IVIS of three treated corneas with corresponding standard deviations (SD) are presented. The eye irritation classes for the OP-KIT and LLBO are visualized by a color gradient. Green color: non-eye irritant; yellow color: mild eye irritant; orange color: moderate eye irritant, and red color: severe eye irritant. For *in vitro* classification according to OECD TG437, only two colors (red and green) are shown, respectively to indicate corrosive/severe eye irritants and non-corrosive/non-severe eye irritants. For *in vivo* classification, a different color gradient is used. Green/yellow color: not labeled; orange color: category 2A and 2B; red color: category 1.

irritation range was assessed to evaluate further the performance of the improved LLBO. IVIS were compared with the *in vitro* (BCOP data from literature) and *in vivo* Global Harmonized System (GHS) classification. Data on opacity was generated with the LLBO and in parallel also data was generated with the OP-KIT opacitometer (Table 9).

The five non-irritating compounds were classified correctly compared to *in vitro* and *in vivo* data using both the OP-KIT and LLBO device. In the ICCVAM DRP of the BCOP test (ICCVAM, 2006), no *in vivo* GHS irritation category was given for L-aspartic acid as the study criteria were not met for assigning an *in vivo* GHS category. However, in the ECETOC database, L-aspartic acid induced opacity in 2 out of 3 animals over the 3-day observation period (ECETOC, 1998). On basis of these findings we classified L-aspartic acid as an *in vivo* GHS category 2A.

Four out of 10 mildly irritating compounds (i.e. cetylpyridiniuim bromide, 1-nitropropane, dimethyl sulfoxide, and methylisobutylketone) were classified correctly using LLBO and OP-KIT compared to the provided *in vitro* classification and *in vivo* GHS. Methyl cyanoacetate showed different results obtained with the LLBO. Two experiments classified methyl cyanoacetate as a mildly irritating compound. The latter is in agreement with the results of the OP-KIT and according to the provided *in vitro* classification. Another two experiments classified this chemical as a moderate eye irritating compound corresponding to the *in vivo* GHS classification (category 2A). Petroleum ether is classified as a mildly irritating chemical according to the *in vitro* classification, but this could not be confirmed using the OP-KIT and LLBO in this study. Both devices classified this chemical as a non-irritating compound. The latter can be due to the volatile characteristics of the chemical. Two chemicals i.e. potassium cyanate and 3-glycidyloxypropyl-trimethoxysilane are classified as mildly irritating compounds using the OP-KIT. This is not in agreement with the classification obtained with the LLBO, which classified both chemicals as moderately (category 2A/2B) irritating. No *in vivo* GHS irritation category was assigned to potassium cyanate in the ICCVAM BRD of the BCOP test (ICCVAM, 2006). However, in the ECETOC database, a mild irritation mainly on the conjunctiva but no opacity was reported over the 3-day observation period (ECETOC, 1998). For this reason we assigned potassium cyanate an *in vivo* GHS irritation category 2B. Tetraaminopyrimidine sulfate and propyl-4-hydroxybenzoate showed different results obtained with the LLBO. One experiment classified both chemicals as mildly irritating compounds. The latter is in agreement with the results of the OP-KIT and according to the provided *in vitro* classification. The other three experiments classified both chemicals as moderate eye irritating compounds using the LLBO.

According to *in vitro* classification from literature, hexadecytrimethyl-ammonium bromide is classified as a moderate (category 2A/2B) eye irritant. The compound was classified as severely (category 1) irritating with LLBO and according to *in vivo* GHS classification. With the OP-KIT, a moderate and severe irritation classification was found. Parafluoraniline is classified as category 1 irritating with LLBO. But according to the OPKIT, parafluoraniline is classified as moderately (category 2A/2B) irritating compared to the provided *in vitro* classification. No *in vivo* GHS category was assigned to parafluoraniline in the ICCVAM DRP of the BCOP test as the criteria were not met to assign an *in vivo* GHS category (ICCVAM, 2006). But as the 6 rabbits showed the highest grade on opacity over the whole cornea with invisible iris during the 3 day observation period, we assigned to parafluoraniline an *in vivo* GHS irritation category 1. It is not expected that with such a strong corneal opacity, the corneas would recover over a 21 day period. The Draize study with parafluoraniline had to be terminated at day 3. Sodium lauryl sulfate (3%) and butyl acetate are not classified by GHS. Both chemicals are classified as moderately irritating

Table 9
In Vitro Irritancy Scores of twenty reference chemicals (Table 2).

Chemical	Study number	IVIS OP-KIT Mean	IVIS OP-KIT SD	IVIS LLBO Mean	IVIS LLBO SD	*In vivo* GHS classification
Anthracene	1	0.7	1.3	8.2	11.1	Not classified
	2	2.7	1.1	13.5	7.6	
Polyethylene glycol 400	1	0.8	0.7	-0.3	0.0	Not classified
	2	1.3	0.4	5.6	4.9	
L-Aspartic acid	1	-0.8	0.1	-13.0	0.2	Category 2A(*)
	2	-1.6	0.2	-11.7	1.0	
2-Mercaptopyrimidine	1	-1.1	1.0	-7.1	4.4	Not classified
	2	0.4	0.2	0.8	1.4	
	3	0.6	0.2	-2.0	0.4	
EDTA, dipotassium salt	1	1.9	1.6	5.6	11.9	Not classified
	2	-0.6	1.7	2.3	2.1	
Cetylpyridinium bromide	1	6.5	1.1	33.9	4.0	Not classified
	2	6.1	1.0	21.4	9.5	
Methyl cyanoacetate	1	8.5	2.0	68.0	3.6	Category 2A
	2	12.2	1.3	88.6	11.0	
	3	10.5	2.8	51.5	6.2	
	4	14.8	2.5	103.6	4.8	
1-Nitropropane	1	7.3	2.0	33.6	16.7	Not classified
	2	6.1	2.4	51.0	2.4	
Dimethyl sulfoxide	1	6.3	4.1	44.2	19.0	Not classified
	2	9.4	0.5	45.0	5.4	
Petroleum ether	1	0.4	0.8	0.0	0.1	Not classified
	2	1.8	1.0	9.8	6.1	
Potassium cyanate	1	17.8	1.0	103.8	8.4	Category 2B(*)
	2	7.5	1.9	87.5	4.8	
Tetraaminopyrimidine sulfate	1	12.5	0.5	96.7	13.9	Not classified
	2	18.0	1.5	107.6	8.0	
	3	8.6	2.3	64.1	6.0	
	4	6.5	3.3	77.8	12.3	
Propyl-4-hydroxybenzoate	1	12.3	3.1	86.4	14.7	Not classified
	2	11.3	0.9	82.6	3.2	
	3	9.0	2.3	68.7	10.9	
	4	18.0	2.1	102.0	4.1	
3-Glycidyloxypropyltrimethoxysilane	1	12.9	1.2	87.7	5.4	Not classified
	2	7.7	3.2	93.3	1.8	
Methylisobutylketone	1	11.4	3.8	65.4	14.6	Not classified
	2	13.2	7.7	61.6	10.5	
Parafluoraniline	1	50.0	18.1	161.1	12.7	Category 1(*)
	2	46.1	2.1	155.5	8.4	
Sodium lauryl sulfate (3%)	1	16.1	2.7	34.7	3.1	Not classified
	2	24.5	7.7	52.4	19.8	
Ethyl acetoacetate	1	32.1	0.5	168.1	10.9	Not classified
	2	28.3	1.2	157.1	3.6	
Hexadecyltrimethylammonium bromide	1	37.2	12.5	134.0	23.9	Category 1
	2	76.9	9.1	212.1	20.8	
Butyl acetate	1	6.4	3.8	36.0	11.3	Not classified
	2	18.5	10.2	64.8	16.0	

In Vitro Irritancy Scores (IVIS) classifications after measurement of opacity with the standard OP-KIT and LLBO, calculated according to Table 4, were compared with high quality *in vivo* GHS. At least two independent experiments were performed for each chemical for the calculations of IVIS OP-KIT and LLBO and for each experiment, the average IVIS of three treated corneas with corresponding standard deviations (SD) are presented. The eye irritation classes are visualized by a color gradient. Green color: non-eye irritant; yellow color: mild eye irritant; orange color: moderate eye irritant, and red color: severe eye irritant, (*) Personal assignment of an GHS category on basis of ECETOC data. For *in vivo* Global Harmonized System (GHS) classification, a different color gradient is used. Green color: not classified; orange color: category 2A or 2B; red color: category 1.

according to *in vitro* classification reported in literature, but this cannot be confirmed by results obtained with OP-KIT and LLBO. Ethyl acetoacetate is classified as moderately irritating according the OP-KIT results and is not classified by GHS. In contrast, according to LLBO data, this chemical is classified as severely irritating to the eyes.

In Fig. 2 an overview is given of the ranking of the twenty compounds on a IVIS scoring scale from −12.3 to 173.1 for LLBO data and from −1.2 to 51.7 for OP-KIT data. It is clear that the chemicals are nicely spread in the LLBO scaling compared to the OP-KIT scaling, which is an indication the LLBO can be used for more sensitive measurements in the mild range.

3.2. Adaptation of cornea holders

Adaptations to the standard cornea holders (Fig. 1B) were made in order to be able to mimic more the real *in vivo* situation when eyes are exposed to compounds.

The liquid category 1 eye irritant DMF was used to determine the suitability of the adapted cornea holder (Fig. 3). It can be concluded that dilution of DMF after 1 min exposure resulted in a dose-dependent decrease of opacity readings using the OP-KIT and LLBO, as expected for dilution of a severe eye irritant, which was statistically significant for dilutions of 33% to 15% of DMF. Furthermore, dilution to 50% of DMF after 20 s exposure gave a more

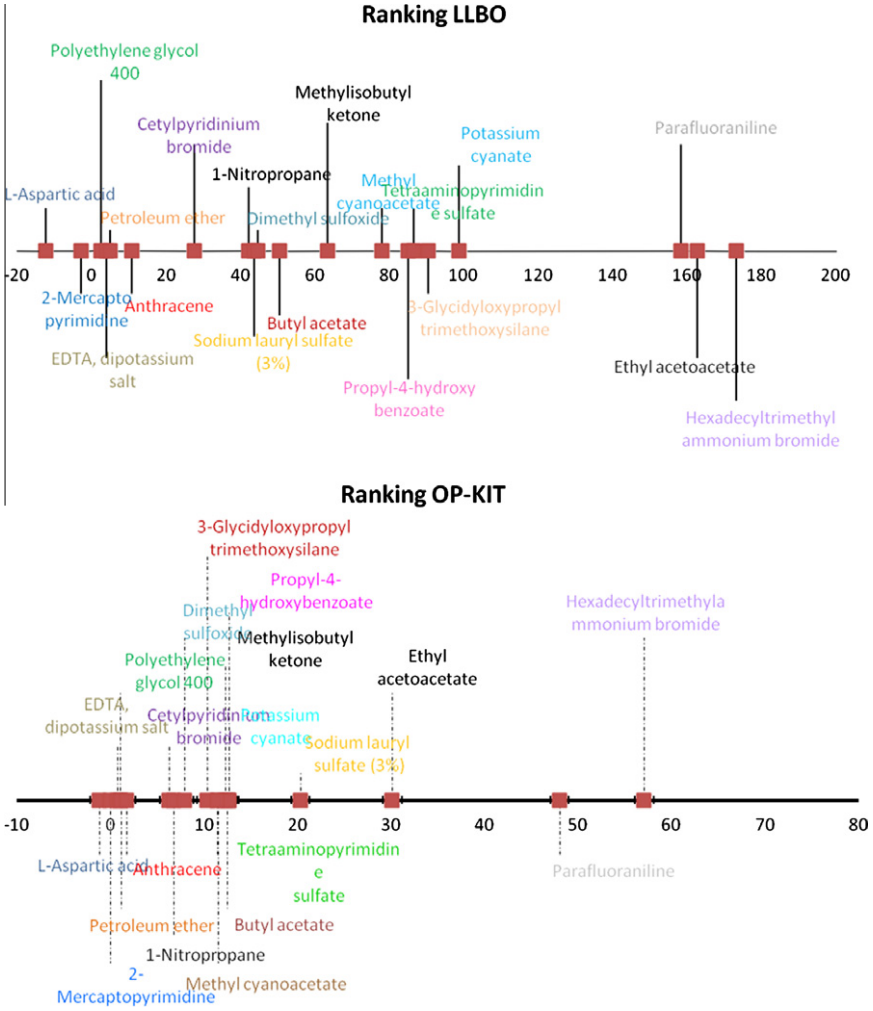

Fig. 2. Ranking scale of *In Vitro* Irritation Scores obtained by the laser light-based opacitometer (LLBO) and OP-KIT for twenty chemicals selected from literature.

pronounced decrease of opacity value in both devices, which was statistically significant ($p = 0.01$ for OP-KIT and $p = 0.002$ for LLBO) compared to 50% dilution after 1 min exposure.

Modifications to the classical BCOP assay should allow a more accurate definition of the eye irritating potential of compounds and a more precise ranking of moderate to mild and non-irritating compounds. For that reason, the suitability of the adapted cornea holder was determined using the liquid moderate (category 2A/2B) eye irritant ethyl acetoacetate (Fig. 4). It can be concluded that dilution of ethyl acetoacetate after 1 min exposure to the compound has no distinct influence on opacity readings using the OP-KIT and LLBO. No steady decrease of average opacity values were noted with increasing dilution. A dilution of 27% of ethyl acetoacetate was statistically significant only for the LLBO ($p = 0.03$).

Another experiment was performed to determine if timing of dilution may have an impact on opacity readings for ethyl acetoacetate (100%) (Fig. 5). After 30 and 10 s of exposure of the corneas to ethyl acetoacetate, the test item was diluted in the adapted cornea holder. After 30 s exposure to ethyl acetoacetate, a slight decrease of opacity readings was observed for a dilution to 67% of test item, with a small increase for a dilution to 33% and again a decrease for 15% dilution of ethyl acetoacetate measured with the OP-KIT. The latter dilution was statistically significant ($p < 0.01$). A small decrease of opacity was observed using the LLBO, which was almost the same for all dilutions. A dilution of 15% using the LLBO was also statistically significant ($p = 0.004$). After only 10 s exposure to the test compound, a slight increase of average opacity readings was observed for a dilution to 67% of ethyl acetoacetate, with a decrease for 33% and 15% of the test compound measured by the OP-KIT. For the LLBO, only dilution to 33% and 15% resulted in a small decrease of opacity readings. The dilution to 15% was statistically significant for both the OP-KIT ($p = 0.002$) and LLBO ($p = 0.035$).

A final experiment was performed to determine if a shorter timing of dilution (5 and 10 s) and if removing of the substance after injection may have an impact on opacity readings for ethyl acetoacetate (Fig. 6). After 10 and 5 s of exposure of the corneas to ethyl acetoacetate, the test item was diluted in the adapted cornea holder. After 10 s exposure to ethyl acetoacetate, a small increase for a dilution to 33% and a decrease for 15% dilution of ethyl acetoacetate was observed for the OP-KIT. The same was observed for measurements using the LLBO. These data were not statistically significant. Furthermore, this was not a confirmation of the previous described results whereby significant data were obtained after 10 s exposure to 15% diluted test substance. After only 5 s exposure to the test compound, a slight increase of average opacity readings was observed for a dilution to 33% of ethyl acetoacetate and a statistically significant ($p < 0.05$) decrease for 15% of the test compound measured by the OP-KIT. The same was observed for the LLBO, but no statistically significant result was obtained. Furthermore, extraction of the substance immediately after injection after 10 s gave a slightly decrease for a dilution of 33% and 15% both for

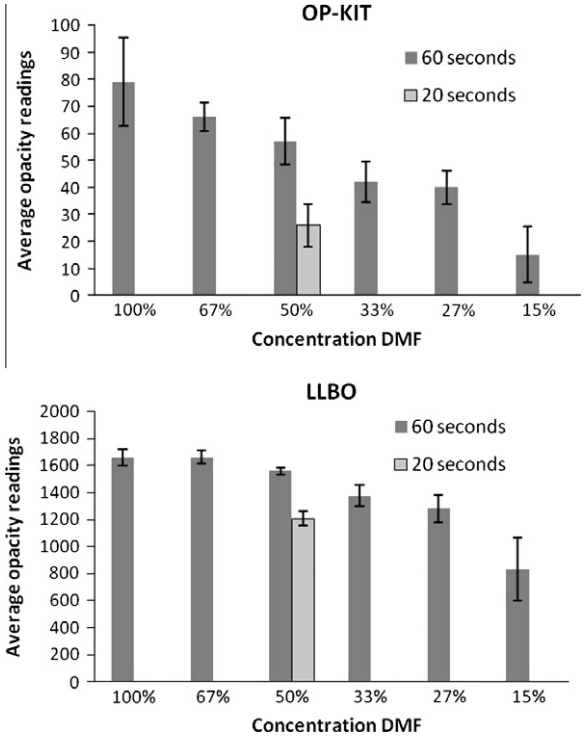

Fig. 3. Dilution of N,N-dimethylformamide (DMF) (severe eye irritant) after 60 s (for all dilutions from 67% to 15%) and 20 s (only for 50% dilution). Opacity was measured using the OP-KIT (upper figure) and laser light-based opacitometer (LLBO, lower figure). Average opacity readings ($n = 3$) and corresponding standard deviations (error bars) are presented on the y-axis, the concentration DMF (%) is shown on the x-axis. A Student's t-Test was performed to determine statistically significancy (*, $p < 0.05$).

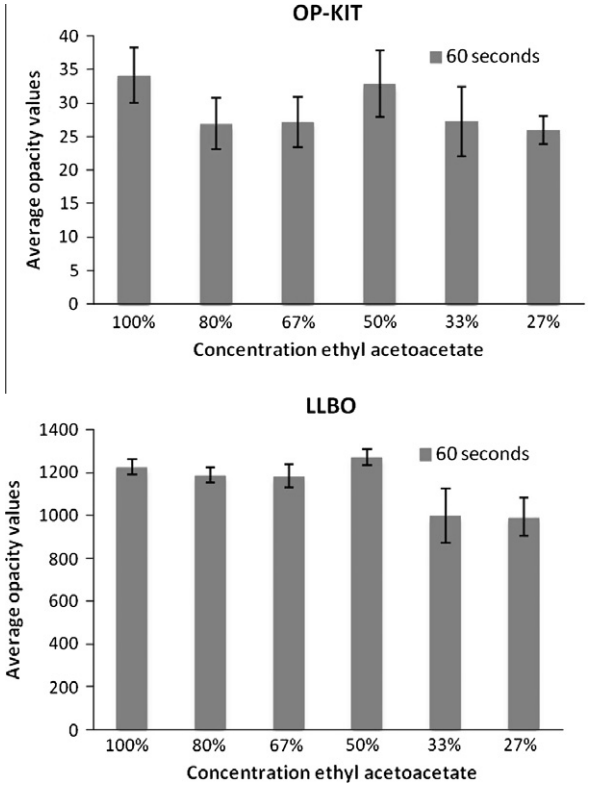

Fig. 4. Dilution of ethyl acetoacetate (moderate eye irritant) after 1 min. Opacity was measured using the OP-KIT (upper figure) and laser light-based opacitometer (LLBO, lower figure). Average opacity readings ($n = 3$) and corresponding standard deviations (error bars) are presented on the y-axis, the concentration ethyl acetoacetate (%) is shown on the x-axis. A Student's t-Test was performed to determine statistically significancy (*, $p < 0.05$).

OP-KIT and LLBO, but these results were not statistically significant.

3.3. Testing an expanded set of formulations

Cosmetic formulations with different irritancy potencies were tested using the LLBO and results were compared with the OP-KIT opacitometer outcome. As the adapted cornea holders used in previous experiments were found to be not suitable, they were not used for these measurements.

All three shampoos (Zwitsal, Fa, and Kandoo) labeled as 'does not sting the eyes' are categorized as mildly irritating products according to the *in vitro* BCOP IVIS data obtained with the OP-KIT device and LLBO (Table 10). The same trend was observed for ranking of the mildly irritating potency for both devices: shampoo Zwitsal < shampoo Fa < shampoo Kandoo. These results are not in agreement with our expectation of 'no' or 'very little' stinging of the eyes as indicated on the shampoo labels. The LLBO was able to better differentiate these shampoo's for the irritating potential than the OP-KIT did.

Simple formulations were made of irritating compounds diluted in artificial tear fluid (Hyabak). Four concentrations (25–50–75–100%) were tested to compare the irritancy ranking using the LLBO and OP-KIT device.

The severe irritant pyridine is categorized as severe to very severe determined by BCOP OP-KIT method (ICCVAM BRDs). *In vivo*, this compound is categorized as category 1 (GHS). In this study, all concentrations of pyridine (0–50–75–100%) are categorized as (very) severely irritating tot the eyes according to the *in vitro* BCOP IVIS data obtained with the OP-KIT device and LLBO (Table 11). For the pure product (100%), the IVIS score was lower compared to the 75% (LLBO and OP-KIT) and 50% (OP-KIT) dilution made of this compound. Only the opacity value, not the permeability value, was responsible for this result, but no explanation could be linked. This phenomenon was more pronounced using the OP-KIT device. No marked decrease in opacity was noted when reducing the pyridine concentration from 100% to 25% which is a characteristic for severe irritation compounds.

γ-Butyrolactone, a moderate irritant, is classified as category 2A (GHS). Furthermore, this compound was mostly classified as moderately irritating using BCOP OP-KIT device, with two exceptions of borderline severely irritating (ICCVAM BRDs). In this study (Table 11), the LLBO and OP-KIT classified γ-butyrolactone as severely irritating for the two highest concentrations (75% and 100%), but one out of two experiments using the OP-KIT device showed a borderline moderate/severely irritating response for 75% dilution of this compound (54.7 ± 2.7). For 50% dilution of γ-butyrolactone, LLBO classified this formulation as severely irritating, whereas the OP-KIT device categorized this as moderately irritating to the eyes. The lowest concentration of γ-butyrolactone was still classified as moderately irritating using the LLBO, with one out of two experiments borderline moderate/severely irritating (125.1 ± 26.5). For the OP-KIT, both repeat experiments classified this compound as mildly irritating. We can conclude here that the LLBO still assigned a higher irritation classification at the two lowest concentrations (25% and 50%) of y-butyrolactone than the OP-KIT did.

The mildly irritating compound 1,2,4-trimethylbenzene was mostly classified as mildly irritating using BCOP OP-KIT device, with two exceptions of borderline moderately irritating (ICCVAM, 2006). *In vivo*, this compound is classified as non-irritating to the

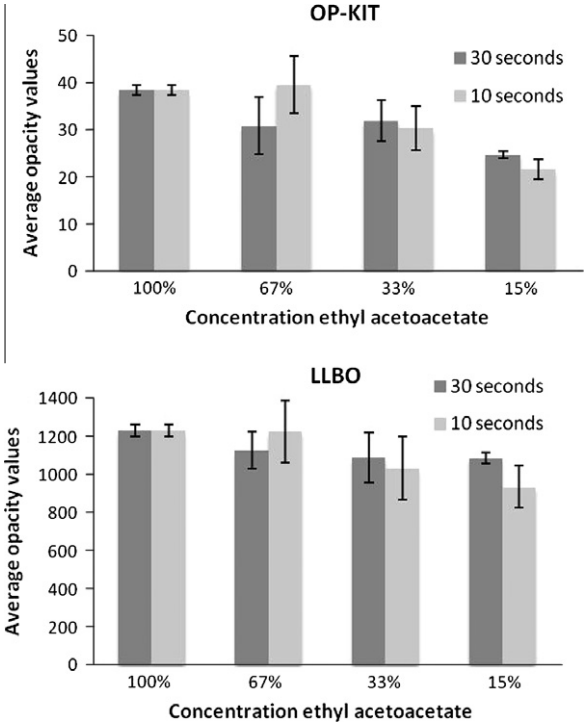

Fig. 5. Dilution of ethyl acetoacetate for 10 (light grey bars) and 30 (dark grey bars) seconds. Opacity was measured using the OP-KIT (upper figure) and laser light-based opacitometer (LLBO, lower figure). Average opacity readings ($n = 3$) and corresponding standard deviations (error bars) are presented on the *y*-axis, the concentration ethyl acetoacetate (%) is shown on the *x*-axis. A Student's *t*-Test was performed to determine statistically significancy (*, $p < 0.05$).

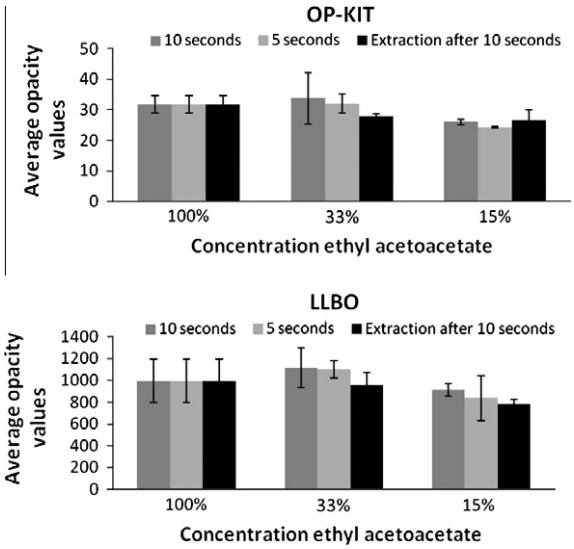

Fig. 6. Dilution of ethyl acetoacetate for 5 (light grey bars) and 10 (dark grey bars) s. Extraction of the compound after 10 s (black bars). Opacity was measured using the OP-KIT (upper figure) and laser light-based opacitometer (LLBO, lower figure). Average opacity readings ($n = 3$) and corresponding standard deviations (error bars) are presented on the *y*-axis, the concentration ethyl acetoacetate (%) is shown on the *x*-axis. A Student's *t*-Test was performed to determine statistically significancy (*, $p < 0.05$).

eyes according to GHS. Here (Table 11), the LLBO classified 1,2,4-trimethylbenzene as moderately irritating for the two highest concentrations (75% and 100%). For the lower concentrations (25% and 50%), both repeat experiments show contrasting classifications. One experiment classified this compound as mildly irritating,

Table 10
In Vitro Irritancy Scores (IVIS) of three commercial kids shampoos. IVIS classifications after measurement of opacity with the standard OP-KIT and laser light-based opacitometer (LLBO), are calculated according to Table 4. Two independent experiments were performed for each shampoo for the calculations of IVIS OP-KIT and LLBO. For each experiment, the average IVIS of three treated corneas with corresponding standard deviations (SD) are presented.

Test item	Study number	IVIS LLBO		IVIS OP-KIT	
		Mean	SD	Mean	SD
Shampoo Zwitsal	1	24.3	7.2	5.7	2.1
	2	25.3	1.6	11.3	1.1
Shampoo Fa	1	43.8	3.6	12.5	3.7
	2	31.3	13.4	13.1	2.4
Shampoo Kandoo	1	55.3	6.9	15.8	3.0
	2	52.1	7.9	16.0	3.2

whereas the other categorized the compound as moderately irritating. A third repeat experiment was performed for the lowest concentration (25%) and categorized the compound for the second time as mildly irritating. Using the OP-KIT device, 1,2,4-trimethylbenzene is categorized as mildly irritating with one exception of the highest concentration (100%) showing contrasting results in the mild and moderate range, as was noted in the ICCVAM BRDs (ICCVAM, 2006). For this concentration, a third repeat experiment was performed. Two out of three experiments classified 100% 1,2,4-trimethylbenzene as mildly irritating. From this data, we can conclude that the LLBO classified the higher 1,2,4-trimethylbenzene concentrations (75% and 100%) in the moderate category while the OP-KIT classified these concentrations in the mild category.

4. Discussion

In the standard BCOP assay, changes in light passage through the cornea have been most commonly assessed with a white light, dual-beam OP-KIT opacitometer. This type of opacitometer provides a center-weighted reading of light transmission by measuring changes in voltage when the transmission of white light passes through the cornea alters. During an in-house validation study of the BCOP assay, we found that that the center-weighted readings may underestimate opacity that develops as spots on the periphery of the isolated cornea. A test substance can induce focal effects when corneas are mounted inaccurately and wrinkles occur leading to non-homogeneous damage to the corneal tissue. In addition, test formulations, when prepared as suspensions, can also induce area-specific injuries. Another observation was that the OP-KIT opacitometer is not sensitive enough to differentiate between non-irritating, mild and moderately irritating compounds and that there is a narrow scale for ranking compounds. Recognizing the limitations of the conventional OP-KIT opacitometer, a prototype laser light-based opacitometer (PLLBO) was developed that uses an adjustable laser beam in combination with a calibrated photocell (Van Goethem et al., 2010).

The main objective of this work was (1) to develop an improved laser light-based opacitometer (LLBO) to allow the analysis of the complete corneal surface and to evaluate its optical characteristics to detect more efficiently opaque spots located along the sides of the excised corneas, and (2) to modify treatment conditions of the corneas in the cornea holders in order to mimic more the real *in vivo* situation. These modifications to the classical BCOP assay should allow a more accurate definition of the eye irritating potential of compounds and a more precise ranking of moderate to mild and non-irritating compounds.

Intensity of laser light can be regulated by an adjustable NDF. Based on preliminary experiments (data not shown), the optimal NDF settings could not be determined. In addition, by using a green

Table 11
In Vitro Irritancy Scores (IVIS) of simple formulations. IVIS classifications after measurement of opacity with the standard OP-KIT and laser light-based opacitometer (LLBO), are calculated according to Table 4. At least two independent experiments were performed for each concentration of a compound for the calculations of IVIS OP-KIT and LLBO. For each experiment, the average IVIS of three treated corneas with corresponding standard deviations (SD) are presented. The eye irritation classes are visualized by a color gradient. Green color: non eye irritant; yellow color: mild eye irritant; orange color: moderate eye irritant, and red color: severe eye irritant.

Test item	Concentration	Study number	IVIS LLBO		IVIS OP-KIT	
			Mean	SD	Mean	SD
Pyridine	100%	1	261.3	9.2	124.9	28.5
		2	259.6	3.1	106.8	2.2
	75%	1	279.8	20.5	174.0	39.5
		2	255.2	19.6	161.4	6.3
	50%	1	248.4	12.1	146.2	24.1
		2	240.2	20.3	133.5	3.9
	25%	1	238.4	5.5	80.1	7.6
		2	222.7	10.7	84.3	12.3
γ-Butyrolactone	100%	1	227.7	17.4	73.2	6.9
		2	235.5	4.3	86.3	14.8
	75%	1	208.4	16.2	63.7	6.5
		2	197.7	15.1	54.7	2.7
	50%	1	195.7	3.2	43.3	0.7
		2	168.3	17.6	36.3	4.7
	25%	1	113.4	18.6	13.8	5.4
		2	125.1	26.5	19.8	4.1
1,2,4-Trimethylbenzene	100%	1	119.6	11.5	30.2	4.1
		2	89.9	10.9	15.2	4.0
		3	80.6	13.1	12.0	3.8
	75%	1	111.4	13.9	18.2	4.3
		2	87.9	22.4	14.3	3.9
	50%	1	82.8	3.7	17.7	9.3
		2	70.4	23.4	12.2	10.2
	25%	1	54.8	11.8	7.1	1.6
		2	86.7	16.6	10.6	4.0
		3	45.4	18.1	5.3	2.0

laser light (LLBO) instead of a red laser light (PLLBO), sensitivity of the LLBO was improved and a larger scaling window was obtained. For that reason a decision was made to set the start values of the NDF at 2000 lux with placement of an empty cornea holder. Without placement of an empty cornea holder, laser light is fully captured by the sensor which is not compatible with the real measurement conditions where treated corneas are measured in a cornea holder.

In this study, the underestimation of peripheral black spots of different diameters due to heterogeneous opacity induction was less pronounced using the LLBO compared to the OP-KIT and PLLBO. The same was true for local opacity induction of the corneas to DMF-absorbent paper at the center or periphery. Again, an improved balanced output of the LLBO was observed compared to the OP-KIT. This was also true for the PLLBO, however, based on the specific prediction models, corneal effects were classified in different categories depending on the location of the spot. A shift occurred from moderate to mild when peripheral opacity was induced with OP-KIT and PLLBO, which was not observed in the LLBO outcome. Inaccurate cornea treatment was reproduced by partially exposing the cornea to methanol and comparing these results with the IVIS induced after full cornea treatment. The weight of the partial treatment was 21.4% for the OP-KIT, compared to 61.5% for the LLBO. An IVIS classification shift from severe to mild was observed for the OP-KIT, and from severe to moderate for the LLBO. The same classification shifts were observed for the PLLBO. However, a slightly lower weight for the partial treatment was observed for the PLLBO (54.4%). These heterogeneous opacity readings support the technical improvement of this new LLBO.

The performance of the improved LLBO was assessed by evaluating the predictive capacity of the new prediction model to discriminate irritants from non-irritants and to compare the IVIS with those of the standard OP-KIT. The prediction model obtained with the LLBO differed from that of the PLLBO in the way that the range for IVIS was smaller in the prototype (from $\leqslant$10 to >100) and opacity was empirically defined as lux/6. When the prediction model of Van Goethem et al. (2010) was applied on the opacity lux values obtained with the LLBO, misclassifications were observed for ammonium nitrate, ethyl-2-methylacetoacetate, and *n*-hexane (data not shown). Applying a lux/7 value and enlarging the scaling window (from $\leqslant$20 to >125) for the new, more sensitive LLBO was found to be an advantage for classification of chemicals in the mild and moderate irritation range.

The eye irritating potential of nine reference chemicals was assessed to evaluate the performance of the improved LLBO. According to OECD TG 437, all IVIS data generated by both the OP-KIT and LLBO are classified correctly as (non)-corrosive/(non)-severe eye irritants compared to the *in vitro* classification. Ammonium nitrate was classified as mild in the OP-KIT and moderately irritating to the eye using the LLBO, indicating a more sensitive reading for the latter device, which was more in correspondence with the *in vivo* GHS classification category 2A. For the chemical 2,6-dichlorobenzoyl chloride, two out of three experiments gave an LLBO IVIS of mild irritant and one experiment of borderline non-irritant, close to mild-irritating, whereas in the standard OP-KIT all three independent experiments classified this chemical as a non-eye irritant. The mildly irritating label defined by the LLBO was more related to the *in vivo* GHS observation in category 2A.

Furthermore, the eye irritating potential of another twenty compounds in the non- to moderate irritation range, based on *in vitro* classification according to BCOP results in literature, was assessed to evaluate further the performance of the improved LLBO. More sensitive outcomes (moderate (LLBO) versus mild

(OP-KIT)) were observed for the following compounds, i.e. methyl cyanoacetate, potassium cyanate, tetraaminopyrimidinesulfate, propyl-4-hydroxybenzoate, and 3-glycidyloxypropyltrimethoxysilane using the LLBO compared to the OP-KIT. Furthermore, more sensitive outcomes (severe (LLBO) versus moderate (OP-KIT)) were observed for parafluoraniline and ethyl acetoacetate using the LLBO compared to the OP-KIT. However, the more sensitive classification of the LLBO was not always in agreement with the *in vivo* classification as 'not classified', which possibly might underestimate the irritation potential of these chemicals, except for methyl cyanoacetate, which was categorized as 'Category 2A'. Furthermore, a better spreading of compounds in the LLBO scaling compared to the OP-KIT scaling was observed, indicating that the LLBO can be used for more sensitive measurements in the mild and moderate range.

In the past, cornea holders were redesigned to maintain normal corneal curvature (Ubels et al., 2002). In this study, adaptations to the standard cornea holders were made in order to be able to mimic more the real *in vivo* situation when eyes are exposed to compounds. To determine the suitability of the adapted cornea holders, concentration series in 0.9% sodium chloride (15–100%) of compounds were tested. The latter is also an ingredient of simulated tear fluid (Kavitha, 2011). Furthermore, the osmolality of 0.9% sodium chloride (292 mOs/kg) approaches that of human tears (303.7 mOs/kg) (Craig et al., 1995). Based on the preliminary experiments in this study, it can be concluded that a significant dilution-related decrease of opacity values was not observed for moderate eye irritants for all dilution concentrations above 27%. The shorter the exposure time, the more the influence of timing of dilution on average opacity values. It is assumed that these compounds directly influence the corneal epithelial cells and induce precipitation of proteins. Industrial companies test their chemicals at concentrations up to 5 à 15% (personal communication). It seems that adaptation of cornea holders is of limited value and only restricted to concentrations up to 15% which mimics more test conditions in industry. In function of exposure time, irritating effects will be seen in depth, but this cannot be measured using both opacitometers.

Cosmetic formulations with different irritancy potencies were tested using the LLBO and results were compared with the OP-KIT opacitometer outcome. As the adapted cornea holders used in previous experiments were found to be not suitable, they were not used for these measurements. A better separation of shampoos for their mildly irritating potential using the LLBO and a difference between the LLBO and OP-KIT in classifying mild and moderate labeled formulations (ICCVAM BRDs) (ICCVAM, 2006) was observed. The LLBO assigned a higher irritation category to some of the concentrations of y-butyrolactone and 1,2,4-trimethylbenzene than the OP-KIT did. A 50% dilution of γ-butyrolactone was classified as severe versus moderately irritating using the LLBO and OP-KIT, respectively. A 25% dilution of γ-butyrolactone was classified as moderate versus mildly irritating using the LLBO and OP-KIT, respectively. For both dilutions, there was one irritation category difference. The same was true for 1,2,4-trimethylbenzene. A 100% dilution of 1,2,4-trimethylbenzene was classified as moderate versus mildly irritating using the LLBO and OP-KIT, respectively. A 75% dilution of 1,2,4-trimethylbenzene was classified as moderate versus mildly irritating using the LLBO and OP-KIT, respectively. Again, there was one irritation category difference between the measurements of both devices.

5. Conclusion

The LLBO showed some promising features which potentially could improve the usefulness of the BCOP test. We observed (1) a better spreading of compounds in the LLBO scaling compared to the OP-KIT scaling, indicating that the LLBO can be used for more sensitive measurements in the mild range, (2) the LLBO underestimated less the peripheral induced opacity compared to the OP-KIT, (3) a better separation of shampoos for their mildly irritating potential using the LLBO, (4) a difference between the LLBO and OP-KIT in classifying mild and moderate labeled formulations, whereby the LLBO assigned a higher irritation category to some of the concentrations of a moderate and mildly irritating compound. We also learned that adaptation of cornea holders is of limited value and only restricted to concentrations up to 15% which mimics more test conditions in industry. In function of exposure time, lesions in deeper structures of the cornea will be seen, but this cannot be measured with the LLBO in its current construction.

The next step is validation of the LLBO, a process by which the reliability and reproducibility of the equipment is to be established, which is needed for international acceptance. Further research should also be performed to expand the LLBO technically to be able to measure depth of injury (DOI). The LLBO has already a camera build in, but research is needed to develop a method to measure DOI.

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