

Appendix A

Publicly Available Protocols for the ICE Test Method

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Appendix A1

INVITTOX Protocol 80. The Chicken Enucleated Eye Test (CEET) – Method of Prinsen

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CHICKEN ENUCLEATED EYE TEST (CEET)

The isolated eye of a chicken is exposed to the test compound and assessed for corneal swelling, corneal opacity and fluorescein retention in order to evaluate the eye irritation potential of the compound.

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NOTE

*The protocol presents the standard operation procedure used in the Home Office UK/EEC Validation Study for Alternatives to the Draize Test. It should be noted that this protocol might need to be modified in light of experience gained in the study. Additional information added in the course of producing this **INVITTOX** protocol, e.g. this note, is presented in italics. In addition, Annex 3, the representative data presented in Table 1 and the list of bibliographical references were not part of the original study SOP. An English language version of the SOP for this test system, as used at TNO, is available on request from Dr. Prinsen.*

Critical Assessment

This protocol was used in an international validation study on alternatives to the Draize eye irritation test for the classification and labelling of chemicals. In this project, nine promising non-animal alternative methods to the Draize Eye Test were selected to undergo final validation. Each of the nine methods was examined by four laboratories and about 60 compounds were tested. The goal of the project was to select those methods which could serve as a valid prescreen or complete replacement of the Draize Eye Test.

The Enucleated Eye Test (EET) with isolated eyes of rabbits has been recognized as a valuable alternative to the Draize eye irritation test, because it represents a test system nearest to the *in vivo* test, without the need to use live animals. However, it is preferable to use slaughterhouse waste tissue as a source of eyes for this procedure. Of the possible eye-donor species, such as the cow, the pig, or the chicken, the latter has been found to be the most suitable for the assessment of eye irritation potential of test materials.

In the Chicken Enucleated Eye Test (CEET), the test compound is applied in one single dose onto the corneas of isolated eyes, which are obtained from slaughterhouse animals after these have been killed. Three parameters are measured to disclose possible adverse eye effects, namely corneal thickness (expressed as corneal swelling), corneal opacity and fluorescein retention of damaged epithelial cells of the cornea. The measurement of corneal swelling in this assay guarantees a highly objective parameter, which enables the investigator to discriminate the damaging effects of test materials very precisely. In combination with the measurement of corneal opacity and fluorescein retention, though assessed by subjective observation, but being accurately measured by the use of a slit-lamp microscope, a reliable evaluation of the eye irritation potential of test materials can be achieved.

Chemicals Tested

Liquid materials are applied with a micropipette in a standard dosing volume of 30 μ l. Pastes may be softened by means of a warm water bath (c. 70°C), collected with the micropipette and applied after cooling down to a lukewarm temperature. This procedure is also applicable when dealing with highly viscous liquids, if they cannot be handled properly at room temperature.

The procedure followed should be noted on the scoring form.

Solids, ground to a fine powder if necessary, are applied by powdering the entire surface of the cornea with a standard amount of 30 mg.

In addition, prior to or during testing, the hydrophobicity or hydrophilicity of liquid materials should be established. This can easily be done by putting some of the compound in a beaker with water and observing whether or not the compound mixes with the water.

Procedure Details

Principle

The test compound is applied onto the corneas of at least three eyes in one single dose. Prior to dosing, each test eye provides its own baseline values for the assessment of corneal effects. One untreated eye serves as a control of the experimental conditions. The reactions of the corneas are examined at regular intervals for up to four hours after treatment. Based on the mean

scores for corneal swelling, corneal opacity and fluorescein retention, an assessment of the eye irritation potential of the compound, ranging from non- to severely irritating, can be made.

Experimental design

In the past, the Enucleated Eye Test has been performed in three of the four laboratories participating in the validation study with this method, using different types of equipment, such as the slit-lamp microscope. Different slit-lamp microscopes and depth measuring devices can give rise to a variation in the swelling ranges obtained. However, ranking of the compounds according to the swelling figures should be similar, regardless of the apparatus.

Since a variation in equipment and in the source of the chickens cannot be avoided, only general directions with respect to these items can be given in the protocol.

Chicken eyes are carefully dissected and placed in a superfusion apparatus using the following procedure:

First the eyelids are carefully removed without damaging the cornea, and a small drop of fluorescein sodium (0.5-2%) is applied to the corneal surface for a few seconds and subsequently rinsed off with isotonic saline at ambient temperature.

N.B.. both 0.5% and 2% concentrations of fluorescein were used by laboratories in the EEC/Colipa study. A 2% concentration is recommended, and is available as Fluorescein sodium BP 2% w/v (Minims, Smith and Nephew Ltd., Romford, England). Usually a flush of 5 ml isotonic saline is sufficient to rinse away the fluorescein that has not been retained by the cornea, but a longer rinsing period may be used if required.

Then, the head with the fluorescein-treated cornea is examined with a slit-lamp microscope (Slit-lamp 900 CN, Haag-Streit AG, Liebefeld-Bern, Switzerland) to ensure that the cornea is not damaged. If undamaged, the eye is further dissected from the head without damaging the eye or cornea. Care is taken to remove the eyeball from the orbit without cutting off the optical nerve too short. The enucleated eye is placed in a clamp (stainless steel clamp available from TNO, Zeist, the Netherlands) with the cornea positioned vertically and transferred to a chamber of the superfusion apparatus (TNO, Zeist). The clamp holding the eye is positioned in such a way that the entire cornea is supplied with isotonic saline from a bent stainless steel tube, at a rate of c. 0.10-0.15 ml/min (peristaltic pump, Desaga STA 131900, Heidelberg, Germany). The chambers of the superfusion apparatus and the saline are temperature-controlled at 32 +/- 1.5°C (water pump, Thermomix 1441, B.Braun Melsungen AG, Melsungen, Germany).

At least four eyes are selected, and, after being placed in the superfusion apparatus, are examined again with the slit-lamp microscope to ensure that they are not damaged. Corneal thickness is accurately measured at the corneal apex, using a Depth Measuring Device (Depth Measuring Attachment no. II for the Haag-Streit slit-lamp microscope) and is expressed in instrument units. Eyes with a corneal thickness deviating more than 10% from the mean value for the eyes, eyes that are unacceptably stained with fluorescein (score higher than 0.5, indicating the cornea to be permeable), or

eyes that show any other signs of damage, are rejected as test eyes and replaced.

After an equilibration period of 45-60 minutes, the corneal thickness of the eyes is measured again to determine the zero reference value for corneal swelling calculations. At time $t = 0$, i.e. immediately after the zero reference measurement, the test substance is applied to the eye. For this purpose, the clamp holding the eye is placed outside the chamber with the cornea facing upwards. Liquid materials are applied in amounts of 0.03 ml from a micropipette, in such a way that the entire surface of the cornea is bathed with the test substance. Solids, ground to a fine powder if necessary, are applied in an amount of 30 mg by powdering the entire surface of the cornea.

After an exposure period of 10 seconds, the corneal surface is rinsed thoroughly with 20 ml of isotonic saline at ambient temperature. The eye in the holder is then returned to its chamber. This procedure is repeated for each test eye. The test substance is tested on at least three eyes; one eye is treated in a similar way with isotonic saline only and serves as a control. The control eye and test eyes are examined at 30, 75, 120, 180 and 240 minutes after treatment, using the criteria and scoring system given in Annex 1. Fluorescein retention is only determined at 30 minutes after treatment. All examinations are carried out with the slit-lamp microscope and are noted on the scoring form given in Annex 2.

N.B.. The dissection process can usually be mastered within a reasonable period, although some training will be required. The number of compounds that can be run on one day will depend on the number of chambers in the superfusion apparatus and on the number of eyes used to test each compound. The superfusion apparatus used at the TNO laboratory had eleven chambers and five eyes are used per compound, allowing two compounds to be run each day, with the eleventh eye serving as the control. Other laboratories use three test eyes per compound and thus can test three compounds per day. The number of tests can easily be increased if more chambers are available.

Annex 3 outlines the classification system used at TNO. A CEET Index was used to analyze data from the participating laboratories in the EEC/Colipa study. This was based on the addition of the maximum mean scores of corneal swelling, corneal opacity and fluorescein retention. The opacity and fluorescein scores were equally weighted in the index when compared to the maximum swelling % obtained. At TNO, the highest swelling observed is usually c. 60%, therefore the max. corneal opacity and fluorescein scores are multiplied by a factor of 20. This was used as the basis for the calculation of provisional CEET scores in the EEC/Colipa study. This way of scoring proved to be quite useful for statistical evaluation of the CEET results and for comparison with in vivo rabbit eye scores. However, it must be noted that the use of different sources for eyes and different thickness measurement devices may result in a variation in the obtained ranges of swelling. In such cases, a correction factor may need to be applied.

ANNEX 1

CRITERIA AND SCORING SYSTEM FOR CORNEAL EFFECTS

The following criteria and scoring systems are applied for the assessment of possible effects:

Corneal swelling

Corneal swelling, expressed as a percentage, is calculated according to the following formula:

$$\frac{\text{corneal thickness at time } t - \text{corneal thickness at time } t = 0}{\text{corneal thickness at } t = 0} \times 100\%$$

The mean percentage of swelling for all test eyes is calculated for the observation time points of 30, 75, 120, 180 and 240 minutes.

Corneal opacity

Opacity degrees of density (the most dense area is used for scoring)

0 = no opacity

0.5 = very faint opacity

1 = scattered or diffuse areas, details of iris clearly visible

2 = easily discernible translucent area, details of iris slightly obscured

3 = severe corneal opacity, no specific details of iris visible, size of pupil barely discernible

4 = complete corneal opacity, iris invisible

The mean corneal opacity value for all test eyes is calculated for the observation time points of 30, 75, 120, 180 and 240 minutes.

Fluorescein retention

0 = no fluorescein retention

0.5 = very minor single cell staining

1 = single cell staining scattered throughout the treated area of the cornea

2 = focal or confluent dense single cell staining

3 = confluent large areas of the cornea retaining fluorescein

The mean fluorescein retention value for all test eyes is calculated for the observation time point of 30, minutes only. If desired, or in case of test substances that have adhered to the cornea, fluorescein retention can be determined at t = 240 min or whenever the test compound is removed.

N.B.. In cases when all the test substance cannot be removed by the initial rinsing, further attempts to rinse it off are made at each observation time point.

Morphological effects

These include "pitting" of corneal epithelial cells, "loosening" of epithelium, "roughening" of the corneal surface and "sticking" of the test substance to the cornea. These findings can vary in severity and they may occur simultaneously. The classification of these findings is subjective to the interpretation of the investigator.

ANNEX 2**TNO NUTRITION - TOXICOLOGY AND NUTRITION INSTITUTE**

ITV/IRR/006 F1
TITLE: SCORING FORM CHICKEN ENUCLEATED EYE TEST (CEET)

TEST COMPOUND: PROJECT NO.: DATE OF TESTING AND SIGNATURE:	LIQUID: YES/NO VISCIOUS: YES/NO WARMED: YES/NO SOLID: YES/NO GROUND: YES/NO HYDROPHILIC/HYDROPHOBIC APPEARANCE:
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EYE NO.	CORNEAL THICKNESS IN INSTRUMENT UNITS AT t =							CORNEAL OPACITY SCORES AT t =						OTHER EFFECTS	FLUORESCIN RETENTION	
	-45	0	30	75	120	180	240	0	30	75	120	180	240		0	30
actual time:->																
1																
sw%																
2																
sw%																
3																
sw%																
4																
sw%																
5																
sw%																
6																
sw%																
init. ³																
mean ¹																
sem ²																

CATEGORY :
CLASSIFICATION :

sw% = corneal swelling percentage; ¹ = mean of the test eyes; ² = standard error of the mean;

³ = initials investigator; C = control eye (to be designated on the form)

REMARKS:

ANNEX 3

TNO CLASSIFICATION SYSTEM APPLIED TO THE CHICKEN ENUCLEATED EYE TEST

1. SEVERITY OF EFFECTS

On the basis of the severity of the observed findings for corneal swelling, corneal opacity and fluorescein retention, the effects are divided into four categories: I = none; II = slight; III = moderate; IV = severe.

Interpretation of corneal swelling, corneal opacity and fluorescein retention and classification into the four categories is done according to the following methodology:

Corneal swelling (TNO classification)

<i>mean corneal swelling %</i>	<i>category</i>
0 - 5	I
6 - 12	II
13 - 18 (>75 min. after treatment)	II
(<75 min. after treatment)	III
19 - 26	III
27 - 32 (>75 min. after treatment)	III
(<75 min. after treatment)	IV
32	IV

NOTE: This classification scheme is only applicable, if the range of swelling percentages obtained is approximately between 0% and 70%. Different slit lamp microscopes and depth measuring devices can give rise to a variation of the swelling range. Therefore, each laboratory must design a category system for corneal swelling using the principles described above, i.e. using the four categories: none, slight, moderate, severe.

Corneal opacity

<i>mean maximum opacity score</i>	<i>category</i>
<i>0.0 - 0.5</i>	<i>I</i>
<i>0.6 - 1.5</i>	<i>II</i>
<i>1.6 - 2.5</i>	<i>III</i>
<i>2.6 - 4.0</i>	<i>IV</i>

Fluorescein retention

<i>mean fluorescein retention score at 30 min after treatment</i>	<i>category</i>
<i>0.0 - 0.5</i>	<i>I</i>
<i>0.6 - 1.5</i>	<i>II</i>
<i>1.6 - 2.5</i>	<i>III</i>
<i>2.6 - 4.0</i>	<i>IV</i>

2. ASSESSMENT OF THE *EX VIVO* EYE IRRITANCY CLASSIFICATION

The *ex vivo* irritancy classification is assessed by reading the irritancy classification that corresponds to the combination of the category obtained for corneal swelling, corneal opacity and fluorescein retention, which are presented in the scheme below:

<i>Classification</i>	<i>Combination of the three categories</i>
<i>A. Not irritating</i>	3 x I 2 x I, 1 x II
<i>B. Slightly irritating</i>	3 x II 2 x II, 1 x I 2 x II, 1 x III 2 x I, 1 x IV ¹ 1 x I, 1 x II, 1 x III ¹
<i>C. Moderately irritating</i>	3 x III 2 x III, 1 x II 2 x III, 1 x IV ² 2 x III, 1 x I ¹ 2 x II, 1 x IV ¹ 1 x II, 1 x III, 1 x IV ¹
<i>D. Severely irritating</i>	3 x IV 2 x IV, 1 x III 2 x IV, 1 x II ¹ 2 x IV, 1 x I ¹ immediate corneal opacity score 3 corneal opacity score 4 severe loosening of epithelium

¹ Combinations of categories less likely to occur.

² Combination can be considered a borderline case between moderately and severely irritating.

Where considerable inter-eye variation is found, the compound should be retested.

3. EC-CLASSIFICATION OF EYE IRRITANTS AND EXTRAPOLATION FROM EX VIVO RESULTS TO EC-CLASSIFICATION

According to Directive 83/467/EEC, Part II (B) of Appendix 6, Dangerous Substances Directive, 5th Adaptation, the *in vivo* irritancy grades are divided into three classes, i.e. non-irritating (NI), irritating (R36) and severely irritating (R41). Extrapolation from the *ex vivo* irritancy grade to the EC-classification will be carried out using scientific judgement, redividing the original four categories into three. The combinations of the three categories that are allowed for each of the three classifications are mentioned in the scheme below.

<i>Classification</i>	<i>Combinations of the three categories</i>
<i>NI = not irritating (combination of A and B)</i>	3 x I 3 x II 2 x I, 1 x II 2 x II, 1 x I 1 x I, 1 x II, 1 x III ¹
<i>R36 = irritating (combination of B and C)</i>	3 x III 2 x II, 1 x III 2 x III, 1 x II 2 x III, 1 x IV 2 x I, 1 x IV ¹ 2 x II, 1 x IV ¹ 2 x III, 1 x I ¹ 1 x II, 1 x III, 1 x IV ¹
<i>R41 = severely irritating</i>	3 x IV 2 x IV, 1 x III 2 x IV, 1 x II ¹ 2 x IV, 1 x I ¹ immediate corneal opacity score 3 corneal opacity score 4 severe loosening of epithelium

¹ Combinations of categories less likely to occur.

The combination of 3 x II can be considered as a borderline case between non-irritating and irritating.

The combination of 2 x III and 1 x IV can be considered as a borderline case between irritating and severely irritating.

Table 1: An example of the use of the TNO classification system to predict irritancy (data from Prinsen and Koëter, 1993)

Test compound	Corneal swelling	Corneal opacity	Fluorescein retention	TNO classification	EC classification
<i>Acetic acid</i>	<i>III</i>	<i>IV</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>
<i>Brij 35</i>	<i>I</i>	<i>I</i>	<i>II</i>	<i>non</i>	<i>NI</i>
<i>Benzalkonium chloride</i>	<i>IV</i>	<i>IV</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>
<i>Dimethyl sulphoxide</i>	<i>I</i>	<i>I</i>	<i>II</i>	<i>non</i>	<i>NI</i>
<i>Sodium fluorescein</i>	<i>I</i>	<i>I</i>	<i>I</i>	<i>non</i>	<i>NI</i>
<i>Glycerol</i>	<i>I</i>	<i>I</i>	<i>I</i>	<i>non</i>	<i>NI</i>
<i>Triacetin</i>	<i>I</i>	<i>I</i>	<i>I</i>	<i>non</i>	<i>NI</i>
<i>Mercury (II) chloride</i>	<i>IV</i>	<i>IV</i>	<i>III</i>	<i>severe</i>	<i>R41</i>
<i>Silver (I) nitrate</i>	<i>II</i>	<i>II</i>	<i>II</i>	<i>slight</i>	<i>NI</i>
<i>NaOH</i>	<i>IV</i>	<i>IV</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>
<i>Toluene</i>	<i>I</i>	<i>II</i>	<i>II</i>	<i>slight</i>	<i>NI</i>
<i>Triethanolamine</i>	<i>I</i>	<i>II</i>	<i>II</i>	<i>slight</i>	<i>NI</i>
<i>n-Hexane</i>	<i>I</i>	<i>I</i>	<i>I</i>	<i>non</i>	<i>NI</i>
<i>Chloroform</i>	<i>III</i>	<i>II</i>	<i>III</i>	<i>moderate</i>	<i>R36</i>
<i>2-Methoxy ethanol</i>	<i>II</i>	<i>III</i>	<i>III</i>	<i>moderate</i>	<i>R36</i>
<i>1-Butanol</i>	<i>IV</i>	<i>III</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>
<i>Acetaldehyde</i>	<i>III</i>	<i>II</i>	<i>III</i>	<i>moderate</i>	<i>R36</i>
<i>2-Butoxyethyl acetate</i>	<i>I</i>	<i>II</i>	<i>II</i>	<i>slight</i>	<i>NI</i>
<i>SDS</i>	<i>III</i>	<i>II</i>	<i>II</i>	<i>severe</i>	<i>R41</i>
<i>Dibutyltin dichloride</i>	<i>IV</i>	<i>III</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>
<i>Tributyltin chloride</i>	<i>IV</i>	<i>III</i>	<i>IV</i>	<i>severe</i>	<i>R41</i>

N.B.. Silver (I) nitrate is a borderline case between NI and R36, 2-methoxy ethanol and acetaldehyde are borderline cases between R36 and R41, and SDS was upgraded from R36 to R41 because of an observed loosening of epithelium.

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***Food and Chem. Toxicol.* 31: 69-76.**

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Appendix A2

Table of ICE Protocols

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Test Method Component	Prinsen (2005)	Prinsen (2000)	Prinsen (1996)	Balls et al. (1995) (INVITTOX Protocol)	Prinsen and Koeter (1993)
Time lapse from abattoir to test	≤ 2hr	≤ 2hr	≤ 2hr	≤ 2hr	≤ 2hr
Age of chicken	7 weeks	7 weeks	7 weeks	not specified	7 weeks
Weight of chicken	2.5-3.0 kg	2.5-3.0 kg	2.5-3.0 kg	not specified	2.5-3.0 kg
Number of eyes/ test material	3	3	5 or 3	at least 3	5
Corneal thickness deviation tolerance	>10% excluded	>10% excluded	>10% excluded	>10% excluded	>10% excluded
Length of equilibration period	45-60 min	45-60 min	45-60 min	45-60 min	45-60 min
Positive control	not included	not included	not included	not included	not included
Negative control	isotonic saline	isotonic saline	isotonic saline	isotonic saline	isotonic saline
Quantity applied	30 mL/30 mg	30 mL/30 mg	30 mL/30 mg	30 mL/30 mg	30 mL/30 mg
Exposure period	10 sec	10 sec	10 sec	10 sec	10 sec
Incubation temp	32±1.5°C	32±1.5°C	32±1.5°C	32±1.5°C	32±1.5°C
Perfusion rate	0.10-0.15 mL/min	0.10-0.15 mL/min	0.10-0.15 mL/min	0.10-0.15 mL/min	0.10-0.15 mL/min
Volume of rinse	20 mL saline	20 mL saline	20 mL saline	20 mL saline	20 mL saline
Slit lamp scope used	Haag-Streit scope	Haag-Streit scope	Haag-Streit scope	Haag-Streit scope	Haag-Streit scope
Evaluation times	0, 30, 75, 120, 180, 240 min PD	0, 30, 75, 120, 180, 240 min PD	0, 30, 75, 120, 180, 240 min PD	0, 30, 75, 120, 180, 240 min PD	0, 30, 75, 120, 180, 240 min PD

Test Method Component	Prinsen (2005)	Prinsen (2000)	Prinsen (1996)	Balls et al. (1995) (INVITTOX Protocol)	Prinsen and Koeter (1993)
Fluorescein observation time (2.0%)	30 min PD	30 min PD	30 min PD	30 min PD	30 min PD
Subjectivity of fluorescein evaluation	0-3 based on #cells retaining fluorescein	0-3 based on #cells retaining fluorescein	0-3 based on #cells retaining fluorescein	0-3 based on #cells retaining fluorescein	0-3 based on #cells retaining fluorescein
Subjectivity of corneal opacity	0-4 based on extent of opacification	0-4 based on extent of opacification	0-4 based on extent of opacification	0-4 based on extent of opacification	0-4 based on extent of opacification
Subjectivity of morphological findings	interpretation of investigator	interpretation of investigator	interpretation of investigator	interpretation of investigator	interpretation of investigator

¹PD = Post dosing