# A Procedure for Application of Eye Irritation Alternative Methods on Cosmetic Ingredients

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**Summary**

*For many years, researchers have sought alternative methods to replace the rabbit Draize eye test for the evaluation of eye irritation that might result from use of cosmetic ingredients. However, no single in vitro assay has been validated. We describe a feasibility study investigating how a combination of in silico, in vitro, and in vivo information could be applied in the assessment of eye irritation hazard. Data from 20 existing and new chemicals (28 concentrations) were used to construct the model, and data from a set of 27 different chemicals were used for verification. The applicability, predictive capacity, severity of misclassifications, and testing costs were evaluated, and a prediction model was established by Linear Discriminant Analysis (LDA). This study demonstrated that a PEIAM can be used to evaluate and compare eye irritation. To fully establish the value of the procedure for application of eye irritation alternative methods (PEIAM), further tests on construction and multi-parameter evaluation need to be conducted.*

*Keywords: alternative methods, in vitro methods, eye irritation test, Linear Discriminant Analysis*

## 1 Introduction

The regulatory assessment of chemical safety is still driven by hazard testing in animals, even though many *in vitro* alternatives, based on different mechanisms, have been studied for evaluation of eye irritation that may result from cosmetic ingredients (Kojima et al., 1995; Eskes, 2005; He, 2006). But in the case of the eye, it is more difficult to imagine a single *in vitro* test replacing the rabbit Draize eye test because of the complexity of reactions that cause eye irritation (Wilhelmus, 2001). However, a statistics analysis, i.e., a Linear Discriminant Analysis (LDA), can help in choosing the procedure for application of eye irritation alternative methods (PEIAM) to enhance the applicability of those *in vitro* methods.

The main purpose of a Linear Discriminant Analysis (LDA) is to gain an understanding of the test systems, as a careful examination of the prediction model that results from the procedure can give insight into the relationship between group membership and the variables used to predict group membership[1].

In this work, we used discriminant analysis to assess 28 chemicals simultaneously, using five *in vitro* assays, HET-CAM, CAM-TBS, IRE, NRU, and FLT. A regression model with a predictive capacity was established based on *in vitro* and Draize test results. A verification study on constructed PEIAM based on applicability, predictive capacity, severity of misclassifications, and testing costs was aimed at devising the best performing strategies to evaluate the eye irritation hazard of cosmetic ingredients.

## 2 Materials, methods

*Test chemicals and in vivo ocular irritation*

The test was performed according to Bagley et al. (1999) and the ECETOC Technical Report 48(2) (1998). The 20 tested chemicals consisted of 12 liquids and 4 solids selected from the ECETOC reference chemicals data bank and assayed at 28 concentrations. *In vivo* data for several of these chemicals were available for more than one concentration, so a total of 28 test substances (one chemical at one concentration) were evaluated in each *in vitro* assay. Where $IC_{50}$ values are reported (e.g., the 3T3 $NRU_{50}$ values), the test substance was considered to be the neat material.

The MMAS data were obtained from the ECETOC Technical Report No. 48. For some of the statistical analyses, the MMAS values were divided into three categories: no/mild irritation MMAS <25; moderate irritation 25≤ MMAS <59; and strong irritant MMAS ≥59.

*Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)*

This test was performed according to Spielmann et al. (1991) and ECVAM DB-ALM INVITTOX Protocol No. 96[2]. The eggs

[1] Kardi Teknomo. Discriminant Analysis Tutorial: http://people.revoledu.com/kardi/tutorial/LDA/

[2] ECVAM DB-ALM: INVITTOX Protocol No. 96. Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM). http://ecvam-dbalm.jrc.ec.europa.eu/view_doc.cfm?iddoc=684 &tdoc=prot

were incubated for 10 days at 37°C at a relative humidity of about 70%, with automatic turning once per hour. After incubation, a portion of each egg shell was removed and a drop of water placed onto the air sack membrane to avoid capillary damage during its removal. The CAM was carefully exposed to 0.1 ml or 0.1 g of test substance, which was washed off with normal saline (37°C) after 30 sec. Each CAM test was observed macroscopically for 5 min for hyperemia, hemorrhage, and coagulation vascular changes. The irritation severity (IS) HET score was calculated based on the time of effect onset. For each chemical, three fertile eggs were exposed to each substance set, and at the same time one fertile egg was exposed to 0.1 ml normal saline as blank control. The average from the three eggs was used as the observed HET-CAM value. Substances were classified in the HET-CAM assay as follows: non-irritant/weak irritant 0-7, moderate irritant 7-15, and severe irritant 15-21.

*Chorioallantoic Membrane-Trypan Blue Staining (CAM-TBS assay)*

This test was performed according to ECVAM DB-ALM INVITTOX Protocol No. 108[3]. On day 10 of incubation, as described above, the portion of egg-shell above the air-space was removed. The CAM was exposed carefully to 0.1 ml of test substance. After the contact period (30 sec), the CAM was treated with 0.5 ml 0.1% trypan blue (1 mM) (Sigma) in phosphate buffered saline (pH 7.4) for 1 min. The dyed CAM was excised and excess pigment was rinsed off with distilled water for 20 sec. The CAM was weighed and incubated in 3 ml of formamide to extract the stain. The absorbance of the extract was measured spectrophotometrically (MPS 2000 spectrophotometer, Shimadzu Co., Ltd, Kyoto, Japan) at 587 nm. The quantity of trypan blue absorbed by the CAM per unit weight was calculated and the $IS_{TB}$ determined. Each substance was used on three eggs, with one blank normal saline control. The $IS_{TB}$ was averaged for three eggs, using the blank control as reference. Substances were classified in the CAM-TBS assay as follows: non-irritant 0-0.1, weak irritant 0.1-0.20, moderate/severe irritant greater than 0.20.

*Fluorescein Leakage Test (FLT)*

This test was performed according to Vinardell et al. (2004) and ECVAM DB-ALM INVITTOX Protocol No. 82[4]. MDCK cells (ATCC no. CCL 34) were stored in liquid nitrogen and cultured in sterile Petri dishes in Eagle's modified minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU penicillin/ml and 50 µg streptomycin/ml. Cells were seeded at a density of $2\times10^5$ per insert on 12 mm Millicell. HA inserts (cellulose acetate; pore size 0.45 µm; Millipore, St Quentin en Yvelines, France) were placed in 24-well plates and grown to confluence for at least 4 days.

Hanks' balanced salt solution (HBSS) was used throughout as washing buffer, as a vehicle for Na-fluorescein, and to dilute test samples to four concentrations (mg/ml) of 0, 20, 50, 100, with one blank control per set that contained an insert with no cells. Every concentration was tested in three wells.

After washing the cells, HBSS (control inserts) or test substances at indicated concentrations were applied to the apical side of the cell monolayer (500 µl/insert) and incubated for 15 min at 37°C, 5% $CO_2$, relative humidity 65%. Cells were washed until test substances were cleared and inserts placed in fresh plates containing 500 µl HBSS. Na-fluorescein (500 µl, 10 µg/ml) was added to each insert and incubated for 30 min, as above.

Inserts were removed and the amount of fluorescein in each well was determined on a Cytofluor TM2300 (Millipore) multiplayer fluorescence reader, at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Fluorescence intensity of the blank control with no cells was set as 100% leakage, and the fluorescence intensity of the 0 mg/ml sample was used as 0% leakage. This first measurement was used to determine the immediate effects (time T0) of the test substance as an impermeability function.

To evaluate recovery of the barrier function, the inserts were washed, placed in new wells with 500 µl fresh medium and incubated for 4, 24, 48, or 72 h. Na-fluorescein was added and leakage evaluated as described above.

The amounts of test substance that caused 20% Na-fluorescein ($FL_{20}$) leakage across the monolayer, compared to cell-free control inserts (maximum leakage), were calculated for each time point (T0, 4, 24, 48, and 72 h). Substances were classified in the FLT assay based on $FL_{20}H_4$ (mg/ml) (the concentration of test material that induced 20% fluorescein passage at 4 h post treatment) as follows: non-irritant/weak irritant, greater than 50; moderate irritant, between 20-50, and severe irritant, less than 20.

*3T3-Neutral Red Uptake (NRU) cytotoxicity assay*

This test was performed according to ECVAM DB-ALM INVITTOX Protocol No. 100[5]. BALB/c 3T3 cells (ATCC clone A31) were cultured in EMEM supplemented with 10% fetal calf serum at 37°C, 5% $CO_2$. Cells were seeded into 96-well plates for 48 h to form a 60-70% confluent monolayer. After removing the culture medium, test substances in culture medium were added to the cells at 100 µl/well; blank controls were EMEM; positive controls were treated with SLS (10 mg/ml) at 100 µl/well. Cells were exposed for 24 ±0.5 h to the test substances over a range of concentrations, with each concentration and control tested in three wells. After 24 ±0.5 h, treatment medium was removed and cells carefully washed three times with HBSS at 200 µl/

[3] ECVAM DB-ALM: INVITTOX Protocol No. 108. CAM-TBS Test. http://ecvam-dbalm.jrc.ec.europa.eu/view_doc.cfm?iddoc=696&tdoc=prot

[4] ECVAM DB-ALM: INVITTOX Protocol No. 82. Fixed Dose Procedure for the Fluorescein Leakage Test. http://ecvam-dbalm.jrc.ec.europa.eu/view_doc.cfm?iddoc=670&tdoc=prot

[5] ECVAM DB-ALM: INVITTOX Protocol No. 100. Neutral Red Bioassay Using BALB/c 3T3 Cells. http://ecvam-dbalm.jrc.ec.europa.eu/view_doc.cfm?iddoc=688&tdoc=prot

well, then incubated with 25 µg/ml Neutral Red (NR) dye medium at 200 µl/well for 3 h. NR medium was discarded and wells carefully washed twice with PBS at 200 µl/well. NR-desorbing solution was added to each well at 100 µl with shaking in the dark for 15 min. The amount of NR absorption was measured by optical density at 540 nm with an ELISA Reader (VERSA, Molecular Devices, Sunnyvale, CA, USA). The concentration of test substance that inhibited NR uptake by 50% (relative to the uptake by the negative control wells) was calculated as $NRU_{50}$. Substances were classified in the NRU assay as $NRU_{50}$ (µg/ml) as follows: non-irritant/weak irritant, greater than 1250; moderate irritant, between 200-1250; and severe irritant, less than 200.

*Red Blood Cell (RBC) haemolysis assay*

This test was performed according to ECVAM DB-ALM INVITTOX Protocol No. 99[6]. RBCs from healthy sheep were obtained from the blood bank of the animal center of Guangdong province. RBCs were diluted in normal saline and centrifuged at 4°C, 1500 x *g* for 10 min. The supernatant was discarded and the process repeated three times. Harvested RBCs were washed with HBSS three times, suspended in HBSS at $4\times10^8$ cells/ml, and stored at 4°C. Test substances were prepared at concentrations ranging from 10 µg/ml to 1000 µg/ml and were mixed with suspended blood cells in a total volume of 1 ml and incubated at 32°C for 30 min. At the end of the incubation, the cell suspension was centrifuged at 1500 x *g* at 4°C for 10 min. Optical density of the supernatant was measured at 540, 575 and 560 nm, with a 723UV-Vis spectrophotometer (Shanghai Precision Instrument Company, China). The percentage of hemolysis was determined by comparing the absorbance (540 nm) of the treated sample with the control sample completely hemolyzed with distilled water (full hemolysis), or with the HBSS negative control. SLS was the positive control, and every substance was tested at six different concentrations. Each concentration was tested with three parallel samples. From the hemolysis results, the dose response curve was determined and the concentration that induced hemolysis in 50% of the cells ($HC_{50}$) was calculated. Protein denaturation was determined from the ratio "$R_x$" of absorbance at 575 and 540 nm in a dual-beam UV-Vis spectrophotometer. $R_1$ was the ratio obtained with the distilled water lysis and $R_2$ was the ratio obtained with the denaturant SLS. The ratio obtained with each test material concentration was Ri. The ratios were used to calculate the hemoglobin denaturation index (DI), where: $DI = R_1 - R_i / R_1 - R_2$. From these values, the ratio of the test substance concentration inducing 50% hemolysis (L) to the denaturation at that concentration (D) (L/D) was calculated to indicate irritation severity. Substances were classified according to the L/D as follows: non-irritants/weak irritants were greater than 50, moderate irritants were 5.0-50, and severe irritants were less than 5.0.

## 3 Results

Statistical comparisons between the MMAS categories and results of the *in vitro* assays are presented in Table 1. Of the 28 chemicals tested, the results of Pearson's chi-square test, McNemar's test, Gamma and Kappa tests all showed significant ranking correlation (Gamma) and class concordance (Kappa) between all these six *in vitro* tests and the Draize test in the classification of chemicals.

The comparison of the predictive capacity between *in vitro* alternative tests and Draize test is presented in Table 2. The result of sensitivity, specificity, and ROC area shows that the order of the predictive capacity to distinguish between non-irritant and irritant is: IRE > CAM-TBS > HET-CAM > 3T3-NRU > FLT > RBC. The order of the predictive capacity to distinguish between non-corrosive and corrosive is: HET-CAM > CAM-TBS > FLT > RBC > 3T3-NRU > IRE.

The LDA predictive models were devised in Table 3. LDA predictive models were devised using the following equations: the MMAS score was calculated as a predicted value from a linear regression, using the standardized coefficients and in vitro test variables. The magnitudes of these coefficients indicate how strongly the *in vitro* test affects the MMAS score.

Based on the results of LDA predictive models and the order of the predictive capacity of the six in vitro tests, we proposed the procedure for application of eye irritation alternative methods (PEIAM) flowchart for eye irritation of cosmetic ingredients. The flowchart is presented in Figure 1.

**Tab. 1: Concordance coefficients between *in vitro* tests and Draize test**

| Coefficients | HET-CAM | CAM-PBS | FLT | 3T3-NRU | IRE | RBC |
|---|---|---|---|---|---|---|
| Pearson $\chi^2$ | 14.943** | 17.388** | 14.982** | 10.173** | 17.477** | 9.334** |
| Pearson correlation | 0.673 | 0.797 | 0.732 | 0.616 | 0.783 | 0.609 |
| Spearman's rank | 0.650 | 0.756 | 0.672 | 0.550 | 0.768 | 0.545 |
| Correction coefficient | 0.724 | 0.798 | 0.719 | 0.598 | 0.798 | 0.577 |

** $p<0.01$

[6] ECVAM DB-ALM: INVITTOX Protocol No. 99. Red Blood Cell Lysis and Protein Denaturation. http://ecvam-dbalm.jrc.ec.europa.eu/view_doc.cfm?iddoc=687&tdoc=prot

**Tab. 2: Comparison of the predictive capacity between *in vitro* alternative tests and Draize test**

| Classification | Method | Sensitivity (%) | Specificity (%) | Roc Area (p) |
|---|---|---|---|---|
| Non-irritant or irritant | IRE | 100.0 | 90.9 | 0.955 (p<0.01) |
| | 3T3-NRU | 70.6 | 72.7 | 0.717 (p<0.01) |
| | HET-CAM | 94.1 | 36.4 | 0.652 (p>0.05) |
| | CAM-TBS | 94.1 | 72.7 | 0.834 (p<0.01) |
| | FLT | 64.7 | 90.9 | 0.778 (p<0.01) |
| | RBC | 52.9 | 90.9 | 0.719 (p>0.05) |
| Non-corrosive or corrosive | IRE | 54.5 | 94.1 | 0.743 (p<0.01) |
| | 3T3-NRU | 54.5 | 100.0 | 0.773 (p<0.01) |
| | HET-CAM | 100.0 | 41.2 | 0.794 (p<0.01) |
| | CAM-TBS | 90.9 | 88.2 | 0.896 (p<0.01) |
| | FLT | 72.7 | 100.0 | 0.864 (p<0.01) |
| | RBC | 63.8 | 100.0 | 0.818 (p<0.01) |

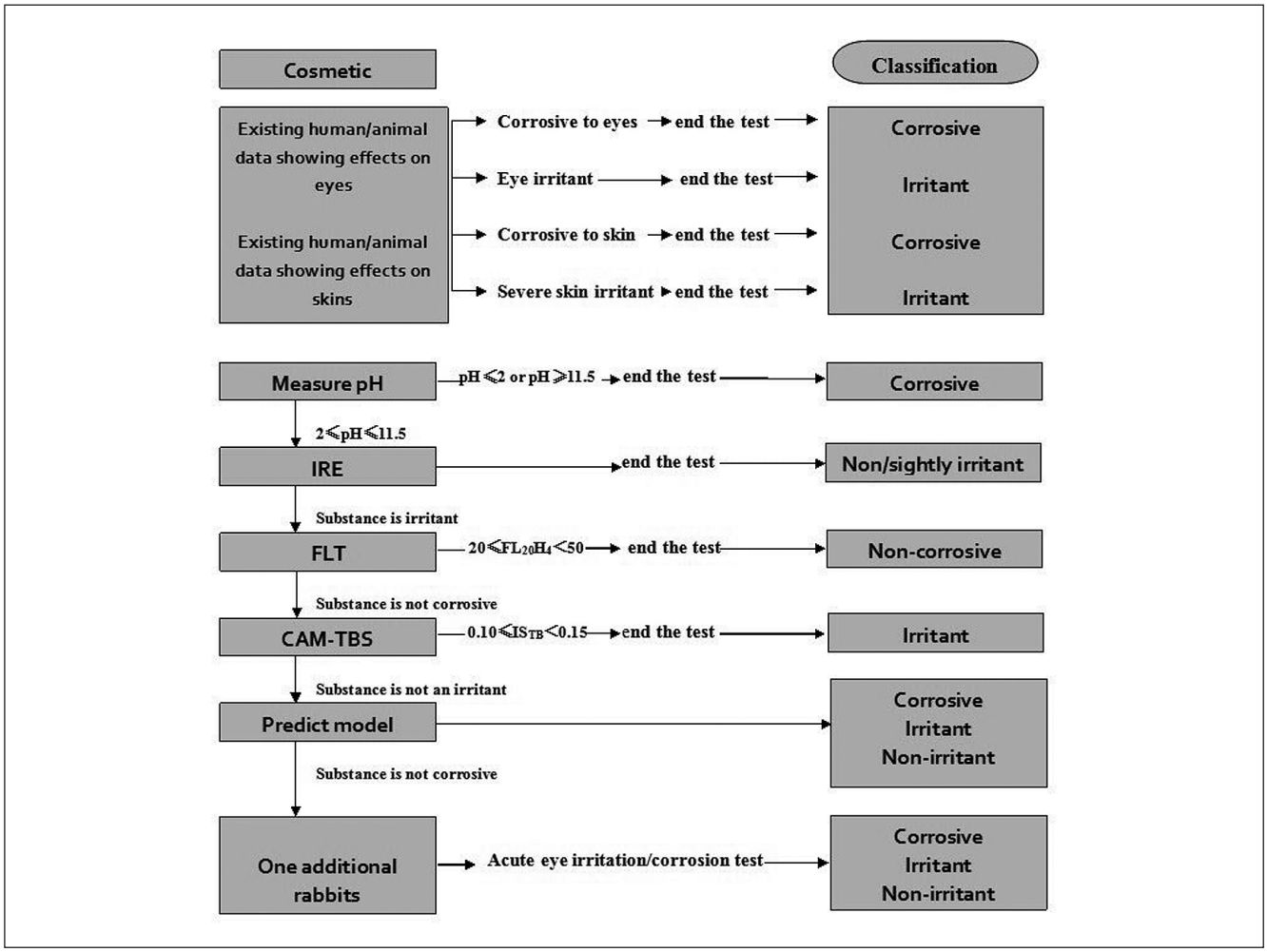


**Fig. 1: The Procedure for Application of Eye Irritation Alternative Methods (PEIAM) flowchart for eye irritation**
Based on the results of LDA predictive models and the order of the predictive capacity of the six *in vitro* tests, we propose the procedure for application of eye irritation alternative methods (PEIAM) flowchart for eye irritation of cosmetic ingredients.

**Tab. 3: LDA predictive models comparing the predictive capacity of *in vitro* alternative tests and Draize test**

| Predict Model | Classification |
|---|---|
| MMAS SCORE=-3.329*CAM-TBS+2.087*FLT+1.823*IRE-5.570 | Non-irritant |
| MMAS SCORE=3.997*CAM-TBS+1.850*FLT+4.719*IRE-11.048 | Irritant |
| MMAS SCORE=7.032*CAM-TBS+5.107*FLT+3.738*IRE-22.584 | Corrosive |

Statistical comparisons between the MMAS categories and results of the *in vitro* assays and PEIAM flowchart on 27 additional chemicals are presented in Figure 2. The predictive accuracy of the fitted values of the PEIAM model was 85.19% (23/27). That is better than results of any single *in vitro* assay, indicating that the model has a good predictive capacity (Fig. 2).

## 4 Conclusion

IRE could be used to screen severely irritating materials. CAM-TBS is simple, fast, sensitive, and inexpensive. It requires no special equipment and is a potential alternative for chemical testing. FLT is mainly used to evaluate the effect of test substances on the barrier function of cells. A constructed PEIAM comprised of IRE, CAM-TBS, and FLT was devised, based on applicability, predictive capacity, severity of misclassifications, and testing costs. It had a good predictive capacity when compared to the results of animal tests, indicating PEIAM can be used to evaluate and compare eye irritation. It is suggested that further tests on the construction and multi-parameter evaluation need to be developed before PEIAM can be used to label and assure the safety of cosmetic ingredients.

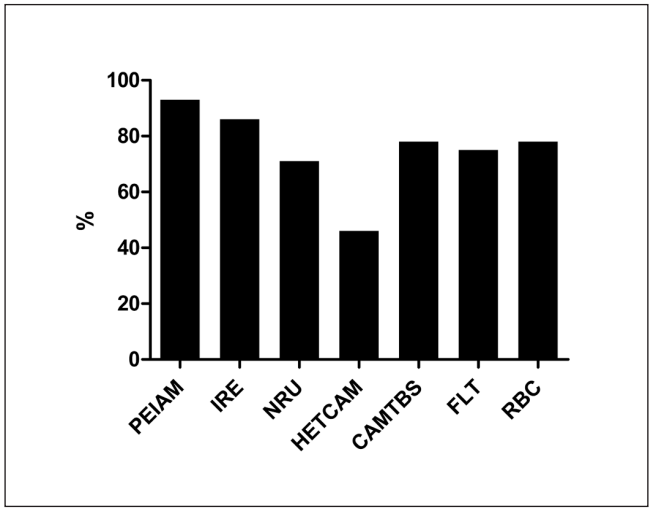


**Fig. 2: Comparison of the predictive capacity between PEIAM and *in vivo* test**
Data from tests on 27 additional chemicals were compared to the fitted values of the model, and the predictive accuracy of the PEIAM was 85.19% (23/27) indicating that the model had a good predictive capacity.

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