

TOXICO-BIOLOGICAL ASSAYS UNIT

**DERMAL IRRITATION ASSAY
DERMAL IRRITATION METHOD *IN VITRO*
(SKINTEX)**

Company: LABORATORIOS DIET ESTHETIC, S.A.

Prod./Sample: CREMA DE ORO

Lot/Reference: -

Petition number: D01944614

CONFIDENTIAL

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DERMAL IRRITATION TEST DERMAL IRRITATION METHOD IN VITRO
(SKINTEX)

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2.- METHOD

The method used in this assay, is described in the instructions manual of the technique, given by INVITRO INTERNATIONAL: "IRRITECTION™ ASSAY SYSTEM INSTRUCTION MANUAL"

Due to the fact that the sample is a cosmetic product, the reactive kit selected has been the Dermal cosmetics reactive kit.

3.- METHOD ABSTRACT

"IRRITECTION ASSAY SYSTEM DERMAL RESPONSE (SKINTEX)"

The test is a in vitro assay, standardized and objective that determines the dermal irritation potential of different products.

The method is based on the test sample application over a membrane of collagen, keratin and colorant which is in contact with a proteinous reagent. When Irritating products get in contact with membrane they may alter it, and provoke a colorant liberation, or/and pass through it and react with the reagent provoking different levels precipitation, according to the sample irritating capacity.

This precipitation is detected as a opacity that is evaluated by a spectrophotometer connected to a computer with the appropriate program that will perform calculations and obtain results.

4.- SAMPLE OF STUDY

Sample: CREMA DE ORO

Reference: -

Petition number: D01944614

5.- ENVIROMENTAL CONDITIONS

The laboratory enviromental conditions during the assay were as follows:

Temperature: 21° C ($\pm 2^{\circ}\text{C}$)

Relative humidity: 55% ($\pm 25\%$)

Air: 15 air renovations per hour and pre-filter at 5 μm

6.- MATERIAL

- IRRITECTION KIT by IN VITRO INTERNATIONAL, containing: Dermal Reagent Powder, Dermal Hydrating Solution, Dermal Blanking Buffer, Dermal Inhibition Check, Dermal Activator, 4 calibrator solutions CAL0, CAL1, CAL2, CAL3, two quality control solutions QC1 and QC2, a 24 holes plate, membrane disks (26 red ones and 22 blanks), wooden sticks for stirring, Watman filter paper, lot Range Specification data sheet certificate
- paper tissue
- Automatic Finnpiette P.C.R. and variable volume pipettes
- One use pipettes tips
- Plastic pincers
- "Eppendorf Repeater TM U780"
- Three precipitate glasses of 100 ml
- 50 ml flask
- Funnel
- Felt-tipped pen
- Portable 506 pHmeter or pH indicator paper
- A Molecular device E-max plate lector
- Software for windows: Irritection program
- ISUZI incubator
- Parafilm
- One use Latex or vinyl gloves

7.- SAMPLE PREPARATION

Four doses are tested: 50 µL, 75 µL, 100 µL y 125 µL.

8.- ASSAY

The Dermal Irritation Assay is a in vitro standardized quantitative test used to predict ocular irritation potential of different cosmetics, pharmaceuticals, raw materials etc. with pH < 9.0. The assay is based on the fact that dermal irritating products provoke alterations and/or denaturalization of collagen, keratin and other dermal protein structures. Dermal Irritation Assay imitates these biochemical phenomena.

The Assay is based on these two compounds:

- Semi-permeable membrane, containing keratin, collagen and a colorant reactive.
- Reactive Solution: Protein macromolecular matrix (globulins) and glycoproteins.

Different amounts of sample are placed in contact with the membrane which is submerged in the reagent solution at 25° C during 24 hours, so that the product will be able to pass through the membrane and get in contact with the protein matrix of the reagent solution.

The reaction between the sample and the reagent's proteins may provoke denaturalization and disintegration of the macromolecular matrix which is detected as a turbidity increase evaluated by spectrophotometer. If there has been alteration in its composition during this process it will also be detected by the colorant indicator liberation which is determined by spectrophotometer. These evaluations are carried out measuring reagent's optical density at 450 nm.

While the sample is being applied on a membrane in contact with the reagent solution, the same quantity of sample is dispensed on another membrane in contact with a blank solution (without protein matrix) that will be used as a control and its optical density values at 450 nm will be subtracted from the result obtained by the sample in the reagent solution.

A standard curve is drawn with the four calibrated substances, whose dermal irritation potential is well known. The comparison between the optical density measurements of the sample and those obtained by the standards allows the HIE (Human Irritancy Equivalent) calculation. By this figure the irritancy of the sample is classified through the scale detailed in chapter 9.

The data analysis and the HIE determination it's carried out by the computer's program connected to the lector

Two quality control solutions given with each kit are tested to ensure the correct assay performance. At least one of the results shall correspond with the specifications described in the intern protocol. The computer's program will calculate and inform if the controls are correct or not and therefor if the assay may or may not be considered as valid, that is QUALIFIED or NOT QUALIFIED.

In order to eliminate the possibility of false positives, a Inhibition test is performed. This test is a positive control, that confirms that any value of Optical density too low is real and not due to an inhibitory effect reaction that may provoked by the sample tested.

9.- EVALUATION CRITERIA AND STUDIED PARAMETERS

Human Irritancy Equivalent (HIE) score	Classification
0.00-0.90	NOT IRRITANT
>0.90-1.20	NOT IRRITANT/ IRRITANT
>1.20-5.00	IRRITANT

10.- RAW DATA

11. - BIBIOLGRAPHY

(1).- JOHM W. DRAIZE y otros. Methods for the Study of irritatiom and toxicity of substances applied topically to the skin and mucous membranes. Journal of Pharmacology and Experimental Therapeutics. 1944.

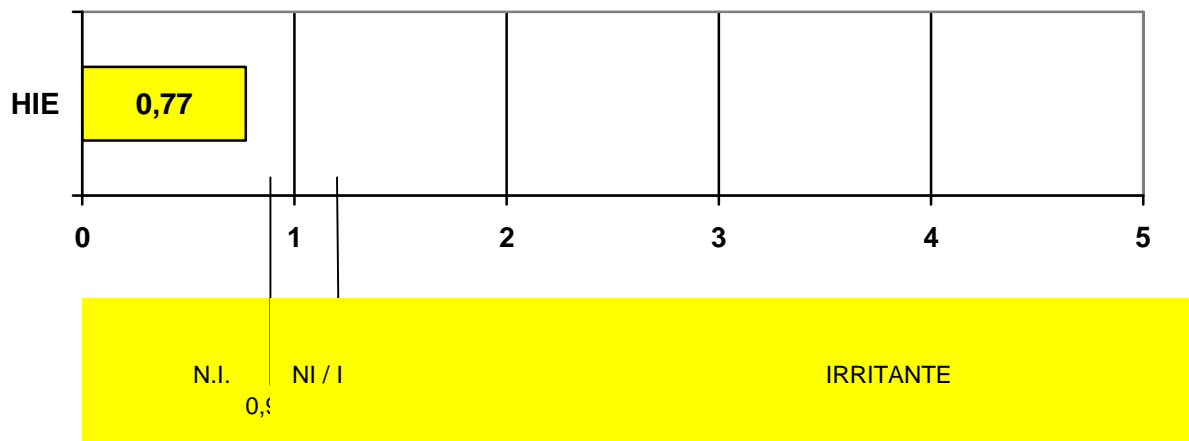
(2).- IRRITECTION™ ASSAY SYSTEM Instruction Manual. In Vitro International 16632 Millikan Avenue, Irvin. Ca 92714. 1996.

(3).- F.H. KRUSZEWSKI

(3).- D. MOUGIN y otros. Correlation Between Rabbit Testing, *In Vitro* Testing and Human Patch Testing In the Evaluation Of Skin Irritation Of Cosmetics. Vy. Y

12.- RESULTS

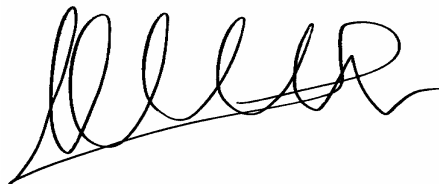
HUMAN IRRITANCE EQUIVALENCE (HIE): **0.77**



N. I. NOT IRRITANT (0 – 0.9)
N.I./I. NOT IRRITANT / IRRITANT (>0.9 – 1.2)
I. IRRITANT (>1.2 – 5)

13.- CONCLUSIONS

Concluded the assay following the method described, the sample " **CREMA DE ORO**" (D01944614) is classified as NOT IRRITANT (HIE=0.77)

A handwritten signature in black ink, consisting of several loops and a long horizontal stroke at the bottom.

Núria Alvarez i Genóher

Biological-Toxicity Assays Department Director

Mataró, 21 August 2008