IFF | LUCASMEYER COSMETICS

TOXICOLOGICAL FILE

GREYVERSE™

ZERO SHADES OF GREY IS ALSO SEXY!

Patented α-MSH biomimetic peptide

-

Stimulates melanogenesis and reduces oxidative stress in hair bulb

-

Reverses hair greying process to progressively recover natural hair color for a younger and more confident look!

PRESERVATIVE FREE

ECO FRIENDLY

CLINICALLY PROVEN

Tolerance/Toxicological File

GREYVERSE™

Lucas Meyer Cosmetics S.A.S.
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OVERVIEW OF GREYVERSE™

ACUTE ORAL TOXICITY IN THE RAT

Study n° TAO423-PH-08/0177 performed by Phycher Bio Développement, Cestas, France.
Compliance: OECD n°423 (adopted December, 17th 2001) and test method B.1 tris of the directive 2004/73/EC

No mortality occurred during the study. No clinical signs related to the administration of Greyverse™ **diluted at 10%** were observed. The macroscopical examination of the animals at the end of the study did not reveal treatment-related changes.

Under the retained experimental conditions, the LD_{50} of Greyverse™ **tested at 10%** is higher than 2000 mg/Kg body weight by oral route in the rat. In accordance with the OECD guideline n°423, the LD_{50} of Greyverse™ **tested at 10%** may be considered higher than 5000 mg/Kg body weight by oral route in the rat.

IN VITRO ALTERNATIVE TO OCULAR IRRITATION TEST – HET-CAM

Study n° 6.02-toxi937-ID-08/0602 performed by IDEA, Martillac, France.
This study is carried out according to the protocol provided in the Journal Officiel de la République Française as of December, 29th 1996.

Under the retained experimental conditions, Greyverse™ tested at **10%** may be classified as **practically non-irritant** according to the adopted scale.

SKIN TOLERANCE – 48-HOURS SINGLE PATCH TEST

Study n° 1.01-48H-ID-08/0602 performed by IDEA, Martillac, France.
The study follows the "Guidelines for the Assessment of Skin Tolerance of Potentially Irritant Cosmetic Ingredients", COLIPA, 1997.

The irritation potential of Greyverse™ tested at **10%** was clinically evaluated following a single contact application under an occlusive patch that was maintained on the skin for 48 hours. The average irritation index obtained is equal to 0.05.

Results obtained under these experimental conditions indicate that Greyverse™ **diluted at 10%** can be considered as **non-irritant** regarding its primary skin tolerance.

IRRITATION AND SENSITIZATION – HUMAN REPEAT INSULT PATCH TEST (HRIPT)

Study n°3.04-ID-08/0602 performed by CTI, Bucharest, Romania.
The study was conducted according to the Good Clinical Practice regulations described by FDA, by the CEE and the Declaration of Helsinki.

Greyverse™ **diluted at 10%** was **applied** under an occlusive patch according to the method of Marzulli-Maibach. Under the conditions of this study, Greyverse™ **(10%) did not induce clinically significant skin irritation nor show any evidence of induced allergic contact dermatitis** in a panel of 55 volunteers.

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Greyverse™ **(10%)** is not contraindicated for usages entailing repeated applications on human skin under conditions appropriate for such products and can be considered as **hypoallergenic**.

MUTAGENIC POTENTIAL *IN VITRO* TEST - AMES TEST

Study n° 08120 performed by International Institute of Biotechnology and Toxicology, Tamil Nadu, India.
The test is conducted in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. adopted 21th July 1997).
The test is performed in accordance with the principles of Good Laboratory Practices.

No cytotoxicity was observed at any dose. No mutagenic response was observed for any of the bacterial strains, at the concentrations tested, with or without the addition of S9.

Under the experimental conditions used, Greyverse™ **tested at 10%** does not show mutagenic nor pro-mutagenic activity in the bacterial reverse mutation test.

PHOTOTOXICITY STUDIES

- In Vitro 3T3 NRU Phototoxicity test
Study n° 6.43-toxi2166-ID-08/0602 performed by IDEA, Martillac, France.
The test was performed in accordance with GLP principles of France, the European Directive 2004/10/EC, the decree dated August 10th, 2004 from the JORF and according to the OECD guideline N°432.

Under the retained experimental conditions, the IC50 (-UV) and the IC50 (+UV) are not reached and the PIF cannot be calculated.
Under the experimental conditions adopted, Greyverse™ **tested at 10%** can be assigned as **non-phototoxic**.

- **Evaluation of the photosensitizing potential in adult volunteers with normal skin**
Study n°3.02-ID-08/0602 performed by CTI, Bucharest, Romania.
The study was conducted according to the Good Clinical Practice regulations described by the CEE and the Declaration of Helsinki.

Greyverse™ **diluted at 10%** was applied on 27 subjects under an **occlusive patch**.
During the induction phase, the repeated applications did not provoke irritation reactions on the irradiated site and the non-irradiated site.
During the challenge phase, no reaction was recorded on the irradiated site and the non-irradiated site.
So, under the conditions of this study, the risks of inducing phototoxicity and/or photallergic reactions with Greyverse™ **(10%)** are minimal.

BIODEGRADABILITY STUDY (CLOSED BOTTLE)

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 301 D permitting the screening of chemicals for ready biodegradability in an aerobic aqueous medium. This method was adopted by the council on 17th July 1992.

After 27 days, the biodegradability of Greyverse™ reached **100.00**%. Greyverse™ was found to be **easily biodegradable**.

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DAPHNIA SP. ACUTE IMMOBILIZATION TEST

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 202 (adopted on 4th April 1984 – last version dated 13th April 2004) and the European Directive (EC) 440/2008 adopted on 30thMay 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).
OECD Guideline 202 describes an acute toxicity test to assess effects of chemicals towards daphnids.

According to the results obtained under the experimental conditions adopted, Greyverse™ can be considered as **non-toxic**, (EC_{50}, 48h) = 34,1 g/L.

FRESHWATER ALGA AND CYANOBACTERIA, GROWTH INHIBITION TEST

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 201 (original adoption: 12th May 1981 – most recently updated: 23th March 2006).
The purpose of this assay is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria.

Under the experimental conditions used, Greyverse™ has **no toxicity to the algal growth rate** (*Pseudokirchnerilla subcapitata)* at concentrations **up to 100 mg/L**; **E_rC_{50} -72h >100 mg/L**.

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TOLERANCE STUDIES

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ACUTE ORAL TOXICITY IN THE RAT

Study n° TAO423-PH-08/0177 performed by Phycher Bio Développement, Cestas, France.
Compliance: OECD n°423 (adopted December, 17th 2001) and test method B.1 tris of the directive 2004/73/EC

INTRODUCTION

The method uses pre-defined dose and the results allow a substance to be ranked and classified according to the Globally Harmonized System.
This procedure is reproducible and uses very few animals.
This method was extensively validated in vivo against LD_{50} data obtained from the literature, both nationally[1] and internationally[2].

PROTOCOL

Animals: Twelve Sprague Dawley female rats were used after an acclimatization period of at least five days. At the beginning of the study, the animals of the treated group weighed between 187 g and 204 g and were 8 weeks old.

Housing: Controlled standardized housing, 3 animals per cage, individually identified.

Dose and administration mode: The animals of the treated group (6 female rats) received an effective dose of 2000 mg/Kg body weight of Greyverse™ **diluted at 10%**, administered by gavage under a volume of 2.03 mL/Kg body weight using a suitable syringe graduated fitted with an oesophageal metal canula.
The animals of the control group (6 female rats) received, according to the same experimental conditions, the control item (distilled water) under a volume of 2 mL/Kg body weight.

Examinations of the animals:
Daily examinations were carried out during 14 days after administration of the test item to identify any behavioural or toxic effects on the major physiological functions.
Periodical examinations: the animals were weighed on day D0 (just before administering the test item) then on D2, D7, and D14. Weight changes were recorded.
Examination at the end of the test: Animals are autopsied at D14. Macroscopic observations were noticed. Only organs likely to be modified in case of acute toxicity were examined. Those presenting macroscopic anomalies can be removed and preserved in view to microscopic examinations.

RESULTS

No mortality occurred during the study.
No clinical signs related to the administration of the test item Greyverse™ **diluted at 10%** were observed.
The body weight evolution of the animals remained normal throughout the study, similar between treated and control animals.
The macroscopical examination of the animals at the end of the study did not reveal treatment-related changes.

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CONCLUSION

In conclusion, the **LD_{50}** of the test item Greyverse™ **diluted at 10%** is **higher than 2000 mg/Kg body weight by oral route** in the rat.
In accordance with the **OECD guideline n°423**, the **LD_{50}** of Greyverse™ **tested at 10% may be considered higher than 5000 mg/Kg body weight by oral route** in the rat.
In accordance with the **Global Harmonized System**, the test item Greyverse™ **diluted at 10% must not be classified in category 4**. No signal word and hazard statement are required.

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IN VITRO ALTERNATIVE TO OCULAR IRRITATION TEST
HET-CAM TEST

Study n° 6.02-toxi937-ID-08/0602 performed by IDEA, Martillac, France.
This study is carried out according to the protocol provided in the Journal Officiel de la République Française as of December, 29th 1996.

OBJECTIVE

The principle of this test is based on a visual observation of the irritant effects (hyperhemia, haemorrhage and coagulation) of Greyverse™ on a vascularized and insensitive Chorioallantoic Membrane (CAM) of Hen's egg, following a single application for five minutes.

This method is an alternative method to animal testing aiming at assessing cosmetic product eye irritation potential.

STUDY RELEVANCE

Luepke showed a good correlation between results obtained with the HET-CAM Test and data obtained using the Ocular Draize Test on rabbit for chemical products. Work sponsored by various European (OPAL, 1991; EEC, 1191; BGA) and American (CTFA) organisms showed that this method is useful to assess the ocular irritation potential of chemicals and formulations.

PROTOCOL

Material and Methods: Chorioallantoic membranes (CAM) of eggs on their **10th day of incubation** were used.

Greyverse™ (10%) was put on the CAM of 4 eggs.
A negative control (solution of lauryl sulfobetaine at 0.05% in physiological serum) and positive controls (solutions of lauryl sulfobetaine at 0.4% and 3.2% in physiological serum) were included in each analysis.

Evaluation of the ocular irritation potential:
Visual observation by a trained person of the irritant effects (hyperhaemia, haemorrhage and coagulation) at $t \leq 30$ sec.; 30 sec. $< t \leq 2$ min.; 2 min. $< t \leq 5$ min.

The score for each egg was the sum of the hyperhaemia, haemorrhage and coagulation notes. The notation of the test element was the arithmetical mean of the scores obtained on 4 eggs, rounded up to one decimal (maximal notation = 21).

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The following scale gave the irritant potential of the test element on the chorioallantoic membrane:

Mean Score (MSc)	Classification
MSc < 1	Practically Non irritant
1 ≤ **MSc** < 5	Slightly irritant
5 ≤ **MSc** < 9	Moderately irritant
MSc ≥ 9	Irritant

RESULTS

Results found for the negative and positive controls allowed us to validate the test.
Greyverse™ (10%): **MSc = 0.**

CONCLUSION

Under the retained experimental conditions, Greyverse™ **(10%)**, tested by the HET-CAM method and according to the JORF classification, is considered as **practically non-irritant**. Regarding its ocular irritant potential, the Greyverse™ product should be **well tolerated at recommended usage (0.1-2%)**.

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PRIMARY SKIN IRRITATION
48-HOURS SINGLE PATCH TEST

Study n° 1.01-48H-ID-08/0602 performed by IDEA, Martillac, France.
The study follows the "Guidelines for the Assessment of Skin Tolerance of Potentially Irritant Cosmetic Ingredients", COLIPA, 1997.

OBJECTIVE

The objective of the study is to check the skin compatibility of Greyverse™ after single application on the external face of the arm under exaggerated experimental conditions (under occlusive patch, for 48 hours).

STUDY RELEVANCE

Cutaneous irritation is a general phenomenon of inflammatory origin which can be defined as a loss of skin integrity. It leads to inflammatory reactions within the dermis and the epidermis that translate into redness (erythema) and oedema.

The human Single Patch Test (occlusive application of Greyverse™ to the skin for 48 hours), allows to check for the absence of cutaneous primary irritation after a single sustained application. Visual scoring is performed according to a pre-established numeric scale.

PROTOCOL

Inclusion Criteria:

- Number of included subjects: 10
- Number of exclusions: 0
- Number of valid cases: 10
- Sex: 8 women and 2 men.
- Age: 21 to 59 years old (Mean = 40.7)
- Skin type: normal skin – There is no dermatological lesion on the experimental area.

Test molecule mode of application:

- *Area*: external face of the arm of the test subject, taking into account the skin appearance and avoiding the areas of friction with clothes
- *Quantity*: 0.02 mL of Greyverse™ **at 10%**
- *Frequency and duration:* single application during 48 hours.
- *Conditions of application*: use of an occlusive patch (Finn-Chambers).

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Modalities of evaluation:

The skin compatibility is based on visual skin examination.

- *Clinical observations*: 30 minutes after removal of the patches, readings were performed by the dermatologist and results obtained in the treated area compared to those obtained for a "negative" control (empty patch).
- *Quantification of cutaneous irritation:* The clinical marking is given according to a numerical scale established depending on the intensity of the irritation phenomena observed (erythema, oedema, dryness, blister, etc.). According to their intensity, the quotation is spread out from 0 to 4.

Analysis of results:

- *Determination of the Average Irritation Index:* the total sum of scores, divided by the number of volunteers, defines the **Average Irritation Index**.
- The ranking of the irritant potential is determined according to the Average Irritation Index obtained as described below:

Average Irritation Index (A.I.I.)	Classification
A.I.I. ≤ 0.20	Non-irritant
0.20 < A.I.I. ≤ 0.50	Slightly irritant
0.50 < A.I.I. ≤ 2.0	Moderately irritant
2.0 < A.I.I. ≤ 3.0	Very irritant
A.I.I. > 3.0	Severely irritant

RESULTS

Results from 10 volunteers have been included in the analysis giving an Average Irritation Index of **0.05** for Greyverse™ **tested at 10%.**
None of the volunteers selected took a treatment contraindicated with the study. No withdrawal of the study happened.

CONCLUSION

Considering the results obtained under these experimental conditions, Greyverse™ **(10%)** shows very good skin compatibility and can be considered as **non-irritant regarding its primary skin tolerance at recommended usage level of 2% and below**.

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IRRITATION & SENSITIZATION – HUMAN REPEAT INSULT PATCH TEST (HRIPT)

Study n°3.04-ID-08/0602 performed by CTI, Bucharest, Romania.
The study has been conducted according to the Good Clinical Practice regulations described by FDA, by the CEE and the Declaration of Helsinki.

OBJECTIVE

This test was done to determine the absence of irritation and sensitization propensities of Greyverse™ following repeated skin applications under an occlusive patch, in healthy adult volunteers.

This test is widely used to evaluate cutaneous sensitization and cutaneous allergenic reactions[3].

STUDY RELEVANCE

Cutaneous allergy is a phenomenon of immune origin that occurs according to three phases:

- Close contact of a foreign allergenic substance with the skin (induction)
- Priming of the immune system following this first contact (rest period)
- Activation of immune reactions following a second exposure of the skin to the allergen (challenge)

All 3 steps are required to document the allergenic potential of a given substance as described by Marzulli and Maibach[4].

PROTOCOL

Participants:

- Number of subjects: 57 volunteers with all type of skin, 42 women and 15 men, 20 to 66 years old.

Test molecule modality of application:

- *Treatment area*: on the back of each subject between the scapulae and waist, adjacent to the spinal mid-line [homolateral site (induction site) and contralateral site (challenge site)]
- *Quantity*: 25 µl of Greyverse™ **tested at 10%**
- *Conditions of application*: use of Haye's chamber occlusive patch
- *Frequency and duration*:
  - Induction phase: patches are applied by the investigator every Monday, Wednesday and Friday until 9 applications. The subjects were instructed to remove the patch 48 hours after application on Sundays. The subjects returned to the Testing Facility and the patch was removed each Tuesday and Thursday then the site was scored by the investigator just prior to the next patch application.

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Dermal responses for the Induction phase of the study were scored according to the following scale:

- = No evidence of any effect
1 = Mild (Pink, uniform erythema covering most of the contact site)
2 = Moderate (Pink-red erythema uniform in the entire contact site)
3 = Marked (Bright red erythema with/without petechiae or papules)
4 = Severe (Deep red erythema with/without vesiculation or weeping)

All other observed dermal sequelae (eg, oedema, dryness, hypo- or hyperpigmentation) were appropriately recorded on the data sheet and described as mild, moderate or severe).

- Rest period: 2 weeks (no applications of the test article).

- Challenge phase: After a rest period of 2 weeks, a Challenge patch was applied to a previously unpatched (virgin) and patched (inductal) test site. The site was scored 30 minutes, 24 and 48 hours after removal. All subjects were instructed to report any delayed skin reactivity that occurred after the final Challenge patch reading.
  Dermal responses for the Challenge phase of the study were scored according to the following criteria of I.C.D.R.G. (The International Contact Dermatitis Research Group):

Score	Interpretation
-	Negative
+?	Doubtful reaction[a] (Slight erythema)
+	Weak (non-vesicular) reaction[b]
++	Strong (oedematous or vesicular) reaction
+++	Extreme (bullous or lucerative)[c]
NT	Not tested
IR	Irritant reaction of different types

[a] ?+ is a questionable faint or macular (non-palpable) erythema and is not interpreted as proven allergic reaction
[b] + is a palpable erythema, suggestive of a slight oedematous reaction
[c] From coalescing vesicles

RESULTS

Only 55 subjects completed this study (2 subjects were discontinued due to a violation of the Protocol).
During the Induction phase, there was 1 mild reaction on 1 volunteer but the study continued without problem through to the Challenge phase.
During the Challenge phase, there was no responses to any subject.

CONCLUSION

Under the conditions of this experiment, Greyverse™ **(10%)** did not induce clinically significant skin irritation nor show any evidence of induced allergic contact dermatitis in human subjects.
Greyverse™ **(10%)** is not contraindicated for usages entailing repeated applications on human skin under conditions appropriate for such products.

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MUTAGENIC POTENTIAL *IN VITRO* TEST – AMES TEST

Study n° 08120 performed by International Institute of Biotechnology and Toxicology, Tamil Nadu, India.
The test is conducted in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. adopted 21th July 1997).
This study is performed in accordance with the principles of Good Laboratory Practices.

OBJECTIVE

The purpose of this assay was to test whether Greyverse™ is mutagenic or pro-mutagenic.

STUDY RELEVANCE

Mc Cann et al. proved the great specificity and sensitivity of this test by establishing the connection between the carcinogenic and mutagenic potential of 300 products. This test is commonly used as a first evaluation test for the mutagenic potential of a test article, in the pharmaceutical, cosmetic, veterinary, as well as chemical industries.

The assay is based on the detection of point mutations (substitution, addition or deletion of one or a few DNA base pairs) or frameshift-mutations in five bacterial strains (*S.typhimurium* TA 98, *S.typhimurium* TA 100, *S.typhimurium* TA 102, *S.typhimurium* TA 1535 and *S.typhimurium* TA 1537) by incubation with one concentration of the product (Greyverse™). These strains have several features that make them most sensitive for the detection of mutations. The mutagenic effect was analyzed in the presence and in the absence of a metabolic system, namely rat liver microsome fraction (S9).

PROTOCOL

Materials: Five bacterial strains (*S.typhimurium* TA 98, *S.typhimurium* TA 100, *S.typhimurium* TA 102, *S.typhimurium* TA 1535 and *S.typhimurium* TA 1537) were used for this assay.

Metabolic Activation system: The S9 mix is prepared from rat liver microsomial fractions and contains metabolic enzymes. The S9 mix is buffered and supplemented with essential co-factors.

Test item: Greyverse™ diluted at 10%.

Method:

Solubility test: As the test item was soluble in sterile distilled water, 0.5 mL of the test item was dissolved in 9.5 mL of sterile distilled water.

Preliminary cytotoxicity assay: it was performed on the five bacterial strains with and without S9. The tested concentrations are: 0.039, 0.078, 0.157, 0.313, 0.625, 1.25, 2.5 and 5 µL/plate. No cytotoxicity of the test item was observed at any of the concentrations employed with and without S9.
Hence, 5 µL/plate was chosen as the highest dose for the main study.

Main study – direct plate incorporation method: test substance dissolved in sterile distilled water was tested at concentrations of 0.05, 0.158, 0.5, 1.58 and 5 µL/plate, with and without S9.

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Based on the negative results obtained from the direct plate incorporation method, a confirmatory assay was performed – pre incubation method. Test concentrations employed were 0.05, 0.158, 0.5, 1.58 and 5 µL/plate, with and without S9.
These two experiments were carried out using each tester strain with plating in triplicates at each concentration.
Control tests were included in these experiments:
Negative controls: Control cultures were treated with the solvent (sterile distilled water), with and without metabolic activation.
Positive controls: known mutagens were used for each strain.

Evaluation of genetic mutations: Following incubation, the number of colonies per plate was counted and recorded.

Data are presented as the number of revertant colonies present per plate (mean ± standard deviation). The R ratio is calculated as follows:

$$R = \frac{\text{Number of revertant colonies in the presence of test item}}{\text{Number of revertant colonies in the absence of test item}}$$

Interpretation of data: All of the following were considered as positive results:

1) A dose-response R increase in the range tested in at least one strain, with or without the metabolic activation system. The mutagenicity is taken into account for a given concentration only when the number of revertants is equal at least to the double of the spontaneous rate of reversion for TA 98, TA 100 and TA 102 (R≥2) and the triple of the spontaneous rate of reversion for TA 1535 and TA 1537 (R≥3).

2) A reproducible R increase at one or more concentration in at least one strain, with or without the metabolic activation system.

Any positive result from the bacterial reverse mutation test indicates that the test item may induce point mutations or reading frame shifts in the bacterial genome.

A negative result indicates that, under the test conditions, the test item is not mutagenic for the bacterial strains tested.

RESULTS

Test controls were in concordance with the expected results:

Positive and negative controls showed absolute numbers of revertant colonies comparable to historical data.

At the concentrations tested, Greyverse™ **(10%)** showed no significant increase in the number of revertant colonies either with or without S9 metabolic activation.

No dose response was observed in any of the tested bacterial strains.

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CONCLUSION

Based on the results obtained in this study, Greyverse™ **(10%)** was found to be **NON-MUTAGENIC** and **NON PRO-MUTAGENIC** under the experimental conditions assayed.

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PHOTOTOXICITY STUDIES

- IN VITRO 3T3 NRU PHOTOTOXICITY TEST

Study n° 6.43-toxi2166-ID-08/0602 performed by IDEA, Martillac, France.
The test was performed in accordance with GLP principles of France, the European Directive 2004/10/EC, the decree dated August 10th, 2004 from the JORF and according to the OECD guideline N°432.

OBJECTIVE

This test was done to document the absence of phototoxicity potential of Greyverse™ with an *in-vitro* test.

STUDY RELEVANCE

This method is an alternative to animal experimentation. The principle is based on the test item cytotoxicity comparison with and without non-cytotoxic UVA dose exposure.
After a cell monolayer (Balb/c 3T3 mouse fibroblasts, clone A31 (ATCC)) incubation with the test item and irradiation with UVA, cytotoxicity is calculated with the help of Neutral Red Uptake. The test item concentrations which give 50 % death cell is determined with and without UVA which allow the photo-irritation factor (PIF) calculation.
This investigation is carried out according to the OECD guideline 432 dated April 13th 2004.

PROTOCOL

Test system: mouse fibroblasts from the Balb/c 3T3 clone 31 (ATCC-CCL 163) cell line.

Culture medium: DMEM medium with 4.5g/L glucose, 4 mM L-Glutamine or stabilized L-Glutamine, 10% complement-free newborn calf serum, 50 IU/mL penicillin and 50 µg/mL streptomycin.

Reference items
Negative control: Hanks saline solution
Positive control: Chlorpromazine solution from 80 to 0.3 µg/mL (CAS number: 69-09-9).

Series definition
Test item: Greyverse™ diluted at 10%.
The test item was tested at 8 concentrations on at least 4 culture wells by assessed concentration. According to the OECD Guideline n°432 recommendation, the maximal concentration tested was 1000 µg/mL followed by 7 dilutions in geometric progression of 2. Dilutions were performed in Hanks balanced salt solution.
Before irradiation, cells were treated with the chemical dilutions for 1 hour (37°C, 5% CO_2).

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Irradiation
The irradiation was performed by a BIO SUN (Vilbert Lourmat) solar irradiator (RMX3W). The used wavelength for the test was 365 nm and the dose applied was 5 J/cm^2. It corresponds to a duration about 20 minutes of exposure for this machine. Concurrently, the non-exposed plates were kept in the BIO SUN chamber protected from the radiation with an aluminum sheet.

Neutral red viability test
Culture plates were incubated with a 50 µg/mL neutral red solution in serum-free culture medium for 3 hours (37°C, 5% CO_2). After incubation, neutral red medium was removed and cells were washed with a buffered saline solution. Neutral red was then extracted with freshly prepared desorption solution (glacial acetic acid 1 vol., ethanol 49 vol., water 50 vol.) for 10 minutes.
Absorbances were read at 540 nm using the desorption solution as blank.

RESULTS CALCULATION AND INTERPRETATION

Results calculation and interpretation:
The cell death rate was calculated for each dilution and each condition (with and without UVA) according to the formula:

$$\% \text{ death rate } \% = 100 - \frac{\text{Average absorbance of treated wells}}{\text{Average absorbance of control wells}} \times 100$$

A curve of percentage cell mortality against the test item concentration was drawn and the test item concentration resulting in 50 % cell mortality (IC_{50}) was determined graphically.
The photo-irritation factor (PIF) was then calculated using the following formula:

$$\textbf{PIF} = IC_{50}\,(-UV) \;/\; IC_{50}\,(+UV)$$

Interpretation
The results were interpreted using the following table:

PIF < 2	Not phototoxic
2 ≤ PIF ≤ 5	Probably phototoxic
PIF > 5	Phototoxic

If it is not possible to calculate an IC50 with or without UVA, this means that no PIF is determined for the test item.

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RESULTS

Test validation
The negative control showed an absorbance higher than 0.4.
The chlorpromazine, positive control, gave an IC_{50} between 0.1 to 2 µg/mL when irradiated and 7 to 80 µg/mL when non-irradiated (PIF higher than 6).
These results allowed us to validate the test.

Results
The test item concentration giving 50 % death cells with UVA cannot be assessed. Mortality never reached 50%.
The test item concentration giving 50 % death cells without UVA cannot be assessed. Mortality never reached 50%.

CONCLUSION

Under the retained experimental conditions, the IC_{50} (-UV) and the IC_{50} (+UV) were not reached and the **PIF cannot be calculated**.
The test item, Greyverse™ diluted at 10% **can be assigned as «non-phototoxic»**.

- EVALUATION OF THE PHOTOSENSITIZING POTENTIAL IN ADULT VOLUNTEERS WITH NORMAL SKIN

Study n°3.02-ID-08/0602 performed by CTI, Bucharest, Romania. Study n°3.02-ID-08/0602 performed by CTI, Bucharest, Romania.
The study was conducted according to the Good Clinical Practice regulations described by the CEE and the Declaration of Helsinki.

OBJECTIVE

This test was done to assess the photoallergic sensitization and photoirritation potential of Greyverse™.

PROTOCOL

Participants: 27 volunteers, 2 males and 25 females, 19 to 60 years old, Phototypes: III

Tested product: Greyverse™ diluted at 10%

Light source and irradiation: Solar light 601 (150 Watt) with filters UG11, UG320 was used

Induction irradiation:
2 sites: with the tested product and without product
-with an irradiation equal to 0.75 MED (spectrum UV-A and UV-B) on D2, D4, D9, D11, D16 and D18

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Challenge irradiation:
2 irradiated sites:
-(UV-A + UV-B) at 0.75 MED
- UV-A at 10 J/cm^2
-the non-irradiated site with product

Induction phase:
On Day 1, approximately 0.02 mL of each tested product was applied to test sites on the subject's back using occlusive patches, and subjects were irradiated at a virgin site of MED determination.
Twenty-four hours later the subjects returned to the Clinic and the patches were removed and each test subject's individual MED was determined.
Twenty-four hours later (forty-eight hours after patching) the subjects returned to the Clinic and both test and control sites were scored for dermal reactivity.
The designated test sites were then irradiated with an UV dose of 0.75 MED. This was repeated for a total of 6 patchings and 6 subsequent irradiations with a rest day betwwen each patching/irradiation visit. There was a 48 hour rest at the week-end. The entire induction took place over a period of 3 weeks.

Challenge phase:
After a 2 weeks rest period, fresh challenge patches were applied to virgin test sites adjacent to the original Induction patch sites.
Twenty-four hours later, the patches were removed from the designated test site. The sites were gently wiped, examined for any dermal reactivity and irradiated with UV-A, and UV-A + UV-B. Following irradiation, the control (Non-Irradiated) patches were removed, the sites were gently wiped and examined for dermal reactivity. The Irradiated and Non-Irradiated sites were scored 24,48 and 96 hours after patch application. The test subjects were asked to report any delayed reactions that might occurred after the final Challenge patch readings.

Evaluation:
Skin responses were scored according to the following scale:

- = No evidence of any effect
0.5 = Barely perceptible (Minimal, faint, uniform or spotty erythema)
1 = Mild (Pink, uniform erythema covering most of the contact site)
2 = Moderate (Pink-red erythema uniform in the entire contact site)
3 = Marked (Bright red erythema with/without petechiae or papules)
4 = Severe (Deep red erythema with/without vesiculation or weeping)

All other observed dermal sequelae (eg, oedema, dryness, hypo- or hyperpigmentation) were appropriately recorded on the data sheet and described as mild, moderate or severe).

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RESULTS

27 subjects satisfactorily completed the test procedure.
During the induction phase, the repeated applications did not provoke irritation reactions on the irradiated test site and the non-irradiated test site.
During the challenge phase, no subject showed any effect on the UV-A + UV-B irradiated test site, on the UV-A irradiated test site and on the non-irradiated test site.
Based on the 27 subjects who completed the study, no clinically significant differences were observed between the Irradiated and Non-Irradiated test sites and no clinically significant evidence of photoirritation or photoallergic dermatitis was observed.

CONCLUSION

Under the conditions of this study, **the risks of inducing phototoxicity and/or photallergic reactions with Greyverse™ (10%) are minimal.**

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BIODEGRADABILITY STUDY (CLOSED BOTTLE)

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 301 D permitting the screening of chemicals for ready biodegradability in an aerobic aqueous medium. This method was adopted by the council on 17th July 1992.

OBJECTIVE

The purpose of this assay was to assess the ready biodegradability of Greyverse™ in an aerobic aqueous medium.

PRINCIPLE OF THE TEST

For this method, the formula of the product and its purity, or relative proportions of major components, should be known so that the Theoretical Oxygen Demand (ThOD) may be calculated. If the ThOD cannot be calculated, the Chemical Oxygen Demand (COD) should be determined.

The solution of the test product in mineral medium (usually 2-5 mg/L) is inoculated with a relatively small number of micro-organisms from a mixed population and kept in completely full, closed bottles in the dark at constant temperature.
Degradation is followed by analysis of dissolved oxygen over a 28-day period and measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation.
The amount of oxygen taken up by the microbial population during biodegradation of the test product, corrected for uptake by the blank inoculum run in parallel, is expressed as a percentage of ThOD or, less satisfactorily COD.

The OECD 301D method evaluates the ability of the inoculum to really degrade the product.

PROTOCOL

The inoculum is derived from the secondary effluent of a treatment plant. The concentration of inoculum introduced in the reaction medium is 2 mL/L.
The concentration of Greyverse™ in reaction medium is 2 mg/L.
The Chemical Oxygen Demand is evaluated and equal to 0.76 mg O_2/mg.
Positive control: Sodium acetate at 10 mg/L.
Analytical method used: Dissolved oxygen measured by electrode method (electrode FDO® 925 WTW).

RESULTS

The biodegradability of Greyverse™ reaches **100.00**% after 28 days.

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CONCLUSION

The pass level for ready biodegradability is 60% of ThOD. This pass value has to be reached in a 10-day window within the 28-day period of the test. The 10-day window begins when the degree of biodegradation has reached 10% ThOD and must end before day 28 of the test.

Based on the results obtained in this study, Greyverse™ **meets the easy biodegradability criteria at a concentration value of 2 mg/L in the reaction medium**. Therefore, Greyverse™ is **easily biodegradable**.

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DAPHNIA SP. ACUTE IMMOBILIZATION TEST

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 202 (adopted on 4th April 1984 – last version dated 13th April 2004) and the European Directive (EC) 440/2008 adopted on 30thMay 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).
OECD Guideline 202 describes an acute toxicity test to assess effects of chemicals towards daphnids.

OBJECTIVE

The objective of this study was the assessment of the acute toxicity effects of the test item Greyverse™ to invertebrates, measured as immobilization of *Daphnia magna*.

STUDY RELEVANCE

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization is recorded at 24 hours and 48 hours and compared with control values. The results are analyzed in order to calculate the EC_{50} at 48h.
EC_{50} is the concentration estimated to immobilize 50 per cent of the daphnids within a stated exposure period. Immobilization: Animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel are considered to be immobilized (even if they can still move their antennae).

PROTOCOL

Material: *Daphnia magma* is used for this assay.
Greyverse™ was tested at 9 concentrations: 0.475 g/L; 0.95 g/L; 1.90 g/L; 3.80 g/L; 7.125 g/L; 14.25 g/L; 23.75 g/L; 35.625 g/L and 42.75 g/L.

Method:

Daphnids (*Daphnia magna*), not older than 24 hours were exposed to 9 concentrations of Greyverse™ under semi-static conditions for a period of 48 hours. The numbered test vessels were completely filled with the test media, the test organisms were added and the vessels were closed with a gas-tight stopper directly afterwards by avoiding air bubbles. No feeding and no aeration occurred throughout the test.

The test media was renewed after 24 hours by transferring the test organisms to new vessels with freshly prepared test media under sterile conditions.

Immobility and abnormal behavior were recorded after 24 and 48 hours. Immobile animals were eliminated from the vessels as soon as they were discovered.

The temperature should be within the range of 18°C - 22°C, and for each single test it should be constant within ±1°C.The beakers were subjected to a light cycle of 16 hours followed by a dark cycle of 8 hours and this light/dark cycles lasted 48 hours.

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Data are analyzed by an appropriate statistical method (e.g. probit analysis, etc.) to calculate the slopes of the curve and the EC_{50} with 95% confidence limit (p = 0.95).

RESULTS

Mortality or immobility of the control: 0%

Greyverse™ conc. (g/L)	Efficacy %
0.475	0
0.95	0
1.90	0
3.80	0
7.125	0
14.25	0
23.75	20
35.625	55
42.75	90

(EC_{50}, 48h) of Greyverse™ is **equal to 34.1 g/L**.

CONCLUSION

According to the results obtained under the experimental conditions adopted, Greyverse™ can be considered as **non-toxic.**

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FRESHWATER ALGA AND CYANOBACTERIA, GROWTH INHIBITION TEST

Study made by Alcycor, Limoges, France.
The test was performed in accordance with OECD Guideline 201 (original adoption: 12th May 1981 – most recently updated: 23th March 2006).
The purpose of this assay is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria.

OBJECTIVE

The purpose of this assay was to assess the effects of **Greyverse™** on the growth of freshwater microalgae and/or cyanobacteria.

PRINCIPLE OF THE TEST

Exponentially growing test algae are exposed to the test substance in batch cultures over a period of normally 72 hours.
The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate. Growth and growth inhibition are quantified from measurements of the algal biomass density as a function of time.
The test endpoint is inhibition of growth, expressed as logarithmic algal biomass increase (average growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified 50 % inhibition of growth rate is determined and expressed as the E_rC_{50}.
In addition, the no observed effect concentration (NOEC) may be statistically determined.

PROTOCOL

Type of specie used: Unicellular green algae, *Pseudokirchneriella subcapitata*.
Culture conditions used:
Culture medium: LC-Oligo medium (pH 7.1 ± 0.1)
Temperature: 21-24°C
Light intensity: light cycle of 16 hours followed by a dark cycle of 8 hours; around 4500 lux
Ventilation: bubbling

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Pre-culture conditions: The algae are incubated for about 3 days under test conditions and used to inoculate the test solutions. This is made to adapt the test alga to the test conditions and ensure that the algae are in the exponential growth phase when used to inoculate the test solutions.

Test conditions:
Duration of test: 72h
Renewal of test solutions: none (static mode)
Growth medium: OECD medium (original medium of OECD TG 201)
Temperature: 21-25°C
Light intensity: constant, 400-700 nm, about 7500 lux
Ventilation: none
Stirring: constant, about 250 rpm
Initial biomass concentration: around 10^4 cells/mL.

This study is made in two steps:

- Screening step: this allows us to determine the concentrations of tested product which inhibit between 5% and 75% of algal growth rate.
  The tests were carried out by using a stock solution of Greyverse™ at 100 mg/L diluted in the test medium.
  Concentrations tested in % of the stock solution: 100%; 35%; 10%; 3.5% and 1%.
- Limit test: when the preliminary test indicates that the test substance has no toxic effects at concentrations up to 100 mg/L, a limit test involving a comparison of responses in a control group and one treatment group (100 mg/L) is undertaken. It could allow us to determine E_rC_{50}-72h; E_rC_{20}-72h; E_rC_{10}-72h and the NOEC.
  [ErCx: Concentration in mg/L of the tested product which causes a reduction of x% of the algal growth rate compared to the control.
  NOEC: Highest tested concentration that did not cause significant inhibition of the algal growth rate compared to the control].

RESULTS

Data processing:
After 72h±2h, measurement of biomass is done for each concentration tested by manual cells counting by microscope. Any abnormal observations are reported.
Specific growth rate and specific growth inhibition rate are calculated for each tested concentration.
ErCx determination is made by using a logistic model based on Hill's equation.
The NOEC is evaluated by statistical model using the Bonferroni t test.

Reference substance used: Potassium dichromate, $K_2Cr_2O_7$ (reference substance for green algae).
Its E_rC_{50}-72h = 1.16 mg/L (value compliant with results previously obtained by the laboratory and between 0.92 mg/L and 1.46 mg/L - acceptable range of sensitivity of algae *P. subcapitata* as defined in standard NF EN ISO 8692: 2012).

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Results of the screening assay:
The percentage of growth inhibition rate is equal to 0 at all the concentration tested.
Greyverse™ has **no toxic effects** at concentrations **up to 100 mg/L**.
Therefore, a limit test is performed at 100 mg/L.

Results of the limit test:
E_rC_{50} -72h > 100 mg/L
E_rC_{20} -72h > 100 mg/L
E_rC_{10} -72h > 100 mg/L
NOEC-72h ≥ 100 mg/L

CONCLUSION

Under the experimental conditions used, Greyverse™ has **no toxicity to the algal growth rate** (*Pseudokirchneriella subcapitata)* at concentrations **up to 100 mg/L**; **E_rC_{50} -72h >100 mg/L**.

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CONCLUSION

Tolerance and safety studies have shown that Greyverse™ **at recommended usage level (2% and below)** presents no risk for cutaneous and/or ocular irritation. Furthermore, Greyverse™ is not mutagenic and has no skin sensitizing properties.

Greyverse™ is also considered as easily biodegradable and non-toxic for the aquatic environment.

In conclusion to these toxicological studies, Greyverse™ is considered as well tolerated and safe for cosmetic purpose at the recommended usage level (2% and below).

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