TOXICOLOGICAL FILE

ACTIVE INGREDIENTS

CAPIXYL™

Hair Fertilizer

Biomimetic peptide combined with a red clover extract

For stronger and thicker hair and lashes

PRESERVATIVE FREE pf

CHINA COMPLIANT

ECO FRIENDLY

in-cosmetics Innovation Zone Best Ingredient Award 2011 BRONZE

LUCASMEYER COSMETICS by IFF

TOLERANCE/TOXICOLOGICAL FILE

CAPIXYL™

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OVERVIEW OF CAPIXYL™

ASSESSMENT OF THE ACUTE ORAL TOXICITY BY USING A 3T3 NRU CYTOTOXICITY ASSAY

Study n° CYTO-CHEM 18.156 performed by BIO-HC, Pessac, France.
This study is performed according to the OECD test guidelines 129 and to EURL ECVAM DB-ALM Protocol n°139.

In the experimental conditions described, the results of the NRU assay indicate an IC50 = 66.17 mg/mL allowing to estimate the acute oral Lethal Dose 50 (LD_{50}) of CAPIXYL™ as **LD_{50} >6000 mg/Kg (> 2000 mg/Kg threshold).**

IN-VITRO ALTERNATIVE TO OCULAR TOLERANCE TEST – HET-CAM

Study n°340505 performed by Eurofins TS, AIX EN PROVENCE, France.
Compliance: Decree published in JO RF of 5 April 1971, modified by the decree of 29 November 1996.

Under the retained experimental conditions, CAPIXYL™ tested at 15% can be classified as **Moderately Irritant**.

SKIN TOLERANCE – 48-HOURS SINGLE PATCH-TEST

Study n°020PT20V09 performed by the Eurofins TS, AIX EN PROVENCE, France.
The study follows the "Guidelines for the Assessment of Skin Tolerance of Potentially Irritant Cosmetic Ingredients", COLIPA, 1997.

The irritation potential of **CAPIXYL™** tested at 25% was clinically evaluated following a single contact application under an occlusive patch that was maintained on the skin for 48 hours. The clinical quotation gave an average irritation index (A.I.I.) of 0.09.

Results obtained under these experimental conditions indicate that topical application of CAPIXYL™ at 25% is **very-slightly irritant** regarding its primary skin tolerance and well tolerated by the skin.

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

Study n°PI27643 performed by the Product Investigations, Inc. Conshohocken, PA.
The study has been conducted according to the Good Clinical Practice defined by FDA (FR 8/08/1978, part V), by the CEE (directives N° 91/507 and III 3976/88 EN of the 11/07/1990) and to the Ministry of Health of the French Republic.

Under the conditions of this HRIPT, **no evidence of dermal irritation or sensitization** for **CAPIXYL™** tested at 15% was observed in a panel of 108 volunteers. These HRIPT data provide evidence for the lack of experimental skin sensitization potential for CAPIXYL™.

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MUTAGENIC POTENTIAL *IN-VITRO* TEST - AMES TEST

Study n° B-00917 performed by Vivotecnia, TRES CANTOS (MADRID), Spain.
The test was performed in accordance with O.C.D.E. Guideline 471 for the Testing Chemicals (Bacterial Reverse Mutation Test adopted 21st July 1997) and the test Method B13/B14 of the Commission Directive 2000/32/EC.
The test was performed according to the European Directive 2004/10/CE and the Good Laboratory Practice (GLP) principle of Spain (RD 1369/2000).

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, CAPIXYL™ tested at 25% did not show mutagenic or pro-mutagenic activities in the bacterial reverse mutation test.

IN-VITRO 3T3 PHOTOTOXICITY TEST

Study n°6.43-toxi6866-ID-10/4334 performed by the Institut Dermatologique d'Aquitaine (IDEA), MARTILLAC, France.
The test was performed in accordance with GLP principles, the European Directive 2004/10/CE, the decree dated August 10th, 2004 from the JORF and according 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. CAPIXYL™, tested at 15% can be assigned as "non-phototoxic".

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 28 days, the biodegradability of CAPIXYL™ reached **100.00**%. CAPIXYL™ was found to be **easily biodegradable**.

DAPHNIA SP. ACUTE IMMOBILIZATION TEST

Study performed by Centre de Transfert de Technologie ODESSOL, 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, CAPIXYL™ can be considered as **non-toxic**; **(EC_{50}, 48h)** is estimated to be equal to **557,62 mg/L**.

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TOLERANCE STUDIES

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ASSESSMENT OF THE ACUTE ORAL TOXICITY BY USING A 3T3 NRU CYTOTOXICITY ASSAY

Study n° CYTO-CHEM 18.156 performed by BIO-HC, Pessac, France.
This study is performed according to the OECD test guidelines 129 and to EURL ECVAM DB-ALM Protocol n°139.

INTRODUCTION

The purpose of the *in-vitro* 3T3 NRU Cytotoxicity assay is to estimate the acute oral toxicity of CAPIXYL™ with a basal cytotoxicity assay in BALB/3T3 murine fibroblasts.
The cytotoxicity is expressed as a dose dependent reduction of the uptake of the vital dye Neutral Red[1] (NR) when measured 48 hours after exposure to increased concentrations of the test item. The test is based on the determination of IC_{50} value, i.e. the concentration of the test item that decreases cell viability by 50%.
The 3T3 NRU Cytotoxicity test is proposed as an *in vitro* method used to estimate an IC_{50} value which in turn is used to predict a LD_{50} value that can serve as the starting dose for the acute oral toxicity test *in vivo* (OECD n°129 Guidance document, 2010[2]).

PROTOCOL

Test system: Mouse fibroblasts cell line, BALB/c 3T3 clone A31, from the ATCC cultured in [DMEM-GlutaMAX-1] medium supplemented with fetal calf serum (10% FCS) and antibiotics ([complete DMEM] medium), maintained under the following conditions:
- temperature: 37°C (± 1°C)
- humidified atmosphere with 5% (± 1%) CO_2.

Test procedure[3]:

Plating: BALB/c 3T3 cells are detached by trypsinization and counted according to standard operating procedures of the laboratory. The cell suspension is seeded into 96-well plates at a density of 3-4 x 10^3 cells per well in [complete DMEM] medium. Two 96-well microplates are used: 1 microplate for the test item and 1 microplate for the positive control tested concurrently in the assay.
After seeding, the 96-well plates are then incubated under the following conditions:
- incubation: 24 hours (± 2 hrs).
- temperature: 37°C (± 1°C)
- humidified atmosphere with 5% (± 1%) CO_2.

Experimental group distribution:
[CAPIXYL™] groups: 9 concentrations (initial test) and 8 concentrations (Main test).
[Negative control] group: [DMEM + 5% FCS] ± solvent
[Positive control] group: 8 concentrations between 10 and 100 µg/mL of Sodium Lauryl Sulfate (SLS) (Main test).

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CAPIXYL™ dilutions: they are prepared on a weight/volume basis using culture medium with 5% FCS. Two separate consecutive experiments are performed to determine the IC_{50} value for each test product.

1. Initial test: range finding experiment

The range of the 9 tested concentrations (6 wells/conc.) is determined adequately by logarithmic serial dilutions from the highest soluble concentration of the test substance in one 96-well plate.
9 concentrations are tested between 0.05 and 150 mg/mL.

2. Main test: IC_{50} determination

The choice of the concentrations to be tested in the main test depends on the results of the initial test. The range of the 8 tested concentrations (6 wells/conc.) is determined adequately around the IC_{50} estimated from the range finder test. The main test should be performed twice.
8 concentrations are tested between 4.20 and 150 mg/mL.

Treatment of cells: 24 hours (± 2 hrs) after seeding, cells are treated with the 8 test-solutions of the test item and/or with the positive control (SLS) concentrations range and incubated for 48 hours (± 0.5h).

Cell viability: after contact with the test item or SLS, the incubation media are removed from plates and neutral red (NR) solution (50 µg/mL) is added into each well. After 180 min (± 10 min) incubation, the NR solution is removed and NR desorb solution (acidified ethanol solution) is added. After 10 minutes (± 1 min) incubation under shaking at room temperature, the optical density (OD) of the NR extract is measured at 540 nm.

Cytotoxicity evaluation:
The optical densities (OD) of each well are corrected by subtracting the mean OD value of respective blanks (n=6) measured in parallel. From the corrected optical densities (OD), the percentage of cell death is calculated for each concentration tested, according to the formula:

$$cell\ death\ \% = \frac{OD_{corr}\,mean[treated\ wells]}{OD_{corr}\,mean[control\ wells]} \times 100$$

The concentration of CAPIXYL™ reducing cell viability to 50% (IC_{50}) is calculated from the dose-response curve using the linear probit-log regression model.

Interpretation of results:
Based on the validation study[4], the LD_{50} value of CAPIXYL™ is estimated from the mean value of IC_{50} values obtained in 2 separate assays, according to the following regression formula:

$$\log DL_{50}\,(mmol/kg) = 0{,}439\,\log CI_{50}\,(mM) + 0{,}621$$

or

$$\log DL_{50}\,(mg/kg) = 0{,}372\,\log CI_{50}\,(\mu g/mL) + 2{,}024$$

Test validation
The 3T3 NRU cytotoxicity test is validated if:

- The positive control (PC) IC_{50} value is within ±2,5 standard deviations (SD) of the historical mean established by the laboratory.
- The PC fitted dose-response curve has an R^2 (coefficient of determination) $\geq$ 0,85.
- The corrected OD_{corr} of each control well ($|VCi|$) is $\leq$ 15% from the mean corrected OD of the negative control.
- For the test item, at least one calculated cytotoxicity value > 0% and $\leq$ 50% viability and at least one calculated cytotoxicity value > 50% and < 100% viability should be present.

These results allow to validate the test.

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RESULTS

Cytotoxicity of CAPIXYL™:
Initial test:
A dose dependent cytotoxic effect was observed from 50 mg/mL concentration.
Highest tested concentration in final test: 150 mg/mL.
Main test:
A dose dependent cytotoxicity was observed in the range of tested concentrations. The percentages of cytotoxicity induced by test concentrations in each assay are collected in the following table:

Conc. (mg/mL)	4.20	7.00	11.66	19.44	32.40	54	90	150
Cytotoxicity assay n°1	2%	7%	10%	16%	24%	37%	64%	100%
Cytotoxicity assay n°2	1%	3%	6%	11%	20%	39%	70%	100%

The IC_{50} value was calculated in each assay using the probit-log [concentration] regression model.
Assay n°1: IC_{50} = 69.81 mg/mL
Assay n°2: IC_{50} = 62.53 mg/mL
IC_{50} (mean of the 2 assays) = 66.17 mg/mL

Cytotoxicity of the positive control:
Main test: Results showed a dose dependent cytotoxicity in the range of tested concentrations.
The IC_{50} value was calculated in each assay using the probit-log [concentration] regression model:
Assay n°1: IC_{50} = 47.63 µg/mL
Assay n°2: IC_{50} = 44.86 µg/mL

LD 50 estimation:
The mean value of IC_{50} obtained (66.17 mg/mL) allows to estimate the LD_{50} value of CAPIXYL™:
LD_{50} = 6566 mg/Kg

CONCLUSION

In the experimental conditions described, the results of the NRU assay indicate an **IC_{50} = 66.17 mg/mL** allowing to estimate the acute oral Lethal Dose 50 (LD_{50}) of CAPIXYL™ as **LD_{50} >6000 mg/Kg (> 2000 mg/Kg threshold).**

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IN-VITRO ALTERNATIVE TO OCULAR IRRITATION TEST
HET-CAM TEST

Study n°389197 performed by Eurofins TS, AIX EN PROVENCE, France.
Compliance: Decree published in JO RF of 5 April 1971, modified by the decree of 29 November 1996.

OBJECTIVE

Visual observation of the irritation effects (hyperemia, hemorrhage, coagulation) of CAPIXYL™ on a vascularized and insensitive Chorion-allantoic Membrane (CAM) of Hen's egg, following a single application for five minutes.
This method is an alternative to animal experimentation 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: Chorion-allantoic membranes (CAM) of eggs on their **10th day of incubation** were used.

CAPIXYL™ (15%) was put on the CAM of at least 4 eggs. A negative control (physiological serum, NaCl to 0.9%) and a positive control (sulfobetaïne 0.8%) were included in each analysis.

Evaluation of the ocular irritation potential:

*Visual observation an rating **(R)*** of hyperemia, hemorrhage, and coagulation were done for each egg at: $t \leq 30$ sec., 30 sec. $< t \leq 2$ min., 2 min. $< t \leq 5$ min

Scale	Classification
R < 1	Practically Non irritant
1 ≤ **R** < 5	Slightly irritant
5 ≤ **R** < 9	Moderately irritant
R ≥ 9	Irritant

Irritation Potential (total R) for each egg = (**R** hyperemia + **R** hemorrhage + **R** coagulation)
*Mean Irritation Index **(MII)*** = total **R / number of eggs**

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RESULTS

Under the retained experimental conditions, the irritation potential index is:

Positive Control: sulfobetaïne 0.8%): Mean Irritation Index (MII) = 12

CAPIXYL™ **(15%) :** Mean Irritation Index **(MII) = 8.9**

CONCLUSION

Under the retained experimental conditions, CAPIXYL™ **(15%)**, tested by the HET-CAM method and according to the JORF classification, is considered as **moderately irritant**. Regarding its ocular irritant potential, the CAPIXYL™ product is **well tolerated at recommended usage (5% and below)**.

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PRIMARY SKIN IRRITATION
48-HOURS SINGLE PATCH-TEST

Study n°335655A01 performed by the Eurofins TS, AIX EN PROVENCE, 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 assess the local skin tolerance of CAPIXYL™ after single application on the skin of the back and under occluded patch during 48 hours, on healthy volunteers.

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 edema.

The human Single Patch Test (occlusive application of CAPIXYL™ 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 subjects: 11.
- Sex: women and/or men.
- Age: 18 to 70 years old.
- Normal skin without any dermatological lesion on the experimental area.

Test molecule mode of application:

- *Area*: back.
- *Quantity*: 0.02 mL of CAPIXYL™ **25%**
- *Conditions of application*: single application of CAPIXYL™, maintained for 48 hours in contact with the skin, with the help of an occlusive patch.

Modalities of evaluation:

- *Clinical observations*: about 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 (patch alone).
- *Quantification of cutaneous irritation:* dermatologists quantified globally the apparition of erythema, papules, vesicles, and blisters on a scale that goes from 0 to 4.

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Analysis of results:

- *Determination of the Average Irritation Index (A.I.I.)*: the total sum of scores, divided by the number of volunteers, defines the **average irritation index (A.I.I.)**, which allows to classify arbitrarily the product as follows:

A.I.I. ≤ 0.08	Non-irritant
0.08 < A.I.I. ≤ 0.16	Very slightly irritant
0.16 < A.I.I. ≤ 0.56	Slightly irritant
0.56 < A.I.I. ≤ 1	Moderately irritant
1 < A.I.I. ≤ 1.6	Irritant
A.I.I. > 1.6	Very irritant

RESULTS

Results from all 11 volunteers have been included in the analysis giving an average irritation index of **0.09** for CAPIXYL™.

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, a single application of CAPIXYL™ **(25%)** can be considered as **very slightly irritant** regarding its primary skin tolerance, would be seen as non-irritant at recommended usage level of 5% and below.

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

Study n°PI27643 performed by the Product Investigations, Inc. Conshohocken, PA.

OBJECTIVE

This test was done to determine the absence of irritation and sensitization propensities of CAPIXYL™ following repeated skin applications under occlusive patch, in healthy adult volunteers.
This test is widely used to evaluate cutaneous sensitization and cutaneous allergenic reactions[5].

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[6].

PROTOCOL

Inclusion Criteria:

- Number of subjects: 112 volunteers, women and/or men, 18 to 84 years old.

Test molecule modality of application:

- *Areas*: on the back of each subject.
- *Quantity*: 200 µl of CAPIXYL™ **15%** on a 24 mm x 27 mm surface.
- *Conditions of application*: CAPIXYL™ under partially occlusive patch
- *Frequency and duration*:
  - Induction period: 3 weeks
  - Rest period: 1 week
  - Challenge phase: 1 week

Assessment Criteria

- ***Clinical criteria or grading Intensity of response***

After each application, the patch is removed and the clinical examination is performed by the investigator 30 minutes later in order to eliminate the pressure and the occlusive effects.
The result of examination is negative if the skin looks normal.

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The clinical examination is made on the back using the following criteria and scale (quotation 0 to 4):

Score	Response	Visible change
0	Absent	None
1	Inflammation: vascular dilation	Faint redness with poorly defined margins
2		Redness with well defined, margins
3	Inflammation: infiltration	Redness plus well defined edema
		Redness plus papules, or vesicles or ulceration

RESULTS

Initial/induction phase:

No responses were notes on any of the 112 subjects who underwent at least one post-application examination. The absence of responses characterizes the product as one which is devoid of clinically significant skin-irritating propensities.

Challenge phase:

Original contact sites: no responses were notes on any of the 105 subjects who participated u=in this phase of the study. The absence of responses characterizes the product as one which is devoid of clinically significant skin sensitizing propensities.

Naïve contact sites: no responses were noted on any of the 105 subjects who participated in the phase of the study. The absence of responses characterizes the product as one which is devoid of clinically significant skin sensitizing propensities.

CONCLUSION

Under the condition of this experiment, CAPIXYL™ **(15%)** was found to be neither a clinically significant skin irritant nor a sensitizer.

CAPIXYL™ **(15%)** 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° B-00917 performed by Vivotecnia, TRES CANTOS (MADRID), Spain.
The test was performed in accordance with O.C.D.E. Guideline 471 for the Testing Chemicals (Bacterial Reverse Mutation Test adopted 21st July 1997) and the test Method B13/B14 of the Commission Directive 2000/32/EC.
The test was performed according to the European Directive 2004/10/CE and the Good Laboratory Practice (GLP) principle of Spain (RD 1369/2000).

OBJECTIVE

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

STUDY RELEVANCE

Mc Cann and 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 *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537) by incubation with five concentration of the product (CAPIXYL™) 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

Material: Five strains of bacteria *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used for this assay.

Metabolic Activation system: The S9 mix is prepared from rat liver microsomial fractions and contains metabolic enzymes (predominantly mono-oxygenase activity mediated via the cytochrome P-450/P-448 system). The S9 mix is buffered and supplemented with essential co-factors.

Methods:

Direct incorporation method (with/without S9 mix): 0.05 to 5 µL of a CAPIXYL™ solution (25%) was tested on each bacteria culture.

Preincubation method (with S9 mix): 0.05 to 5 µL of a CAPIXYL™ solution (25%) was tested on each bacterial culture.

Two controls were included in experiments:

Negative control: Absolute negative control (spontaneous reverse rate). Control cultures were treated with solvent.

Positive control: known mutagens were used for each strain.

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Evaluation of genetic mutations: Following incubation (48 hours at 37°C), the number of colonies per plate was counted with an automatic device.

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

$$\mathbf{R} = \frac{\text{Number of revertant colonies in the presence of CAPIXYL™}}{\text{Number of revertant colonies in the absence of CAPIXYL™}}$$

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.

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 strain tested.

RESULTS

Test controls were in concordance with the expected results:

No cytotoxic effect was observed in the presence of CAPIXYL™ **25%**

Sterility tests showed no contamination of the S9 fraction used as a metabolic activation system

All positive controls performed showed a valid ratio (R) above 2.5

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

At all concentrations tested, CAPIXYL™ showed no significant increase (R≥ 2.5) in the number of revertant colonies either with or without S9 metabolic activation.

No dose response was observed in none of the tested bacterial strains.

CONCLUSION

Based on the results obtained in this study, CAPIXYL™ **(25%)** was found to be **NON-MUTAGENIC** and **NON PRO-MUTAGENIC** under the conditions used for this bacterial reverse mutation test.

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IN-VITRO 3T3 NRU PHOTOTOXICITY TEST

Study n°6.43-toxi6866-ID-10/4334 performed by the Institut Dermatologique d'Aquitaine (IDEA), MARTILLAC, France.
The test was performed in accordance with GLP principles, the European Directive 2004/10/CE, the decree dated August 10th, 2004 from the JORF and according the OECD guideline N°432.

OBJECTIVE

This test was done to document the absence of phototoxicity potential of CAPIXYL™ 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 (murine embryon fibroblast balb/c 3T3 clone 31 – ATCC-CCL 163) 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 witch allow the photo-irritation factor (PIF) calculation.
Analysis by the COLIPA "phototox" version 2 software is also performed (PIF and Mean Photo-Effect (MPE) determination).
This investigation is carried out according to the OECD guideline 432 dated April 13th 2004.

PROTOCOL

Test systems: mouse embryo fibroblasts from the Balb/c 3T3 clone 31 (ATCC-CCL163) cell line.
Radiation sensitivity of cells is determined according to the standard working instruction IT IN VITRO 23. Cells under 5 J/cm^2 UVA present 0% mortality.

Reference items:
Negative control: test item diluent.
Positive control: chlorpromazine solution from 80 to 0.3 µg/ml – CAS number: 69-09-9.

Series definition: Each sample is tested at 8 concentrations on at least four culture wells by assessed concentration.
In agreement with the OECD guideline 432 recommendation, the maximal concentration tested is 1000 µg/ml or the concentration corresponding to the limit of solubility of the test item in its diluents, followed by 7 dilutions in geometric progression of 2.
The pH of the higher concentration is between 6.5 and 7.8.
Before irradiation cells are treated with the chemical dilutions for 1 hour (37°C, 5% CO_2).

Irradiation
The irradiation is performed by a BIO SUN solar irradiator. Plate without irradiation, is kept in the BIO SUN chamber protect from the radiation with an aluminum foil sheet.

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Neutral Red Uptake test
Culture plates are incubated with 100µL of a 50 µg/mL neutral red solution in serum free culture medium for 3 hours. After incubation, neutral red medium is removed and cells are washed with balanced saline solution. Neutral red is the extracted with freshly prepared desorption solution for about 10 min.
Absorbances are read at 540 nm in front of desorption solution as blank.

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

$$\textbf{\% death rate} = \frac{\text{Mean Abs negative control} - \text{Mean Abs Test item}}{\text{Mean Abs negative control}} \times 100$$

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

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

The calculation of PIF is also performed using the COLIPA "phototox" version 2.0 software provided by the OECD. The software report is provided at the end of the report.

The differences observed between the 2 methods of determination of the IC_{50} come from the calculating methods used: a simple graphic determination on the one hand and computation software based on a sophisticated mathematical model on the other hand.

MPE determination
The mean photo-effect (MPE) is based on comparison of the complete concentration response curves. It is defined as the weighted average across a representative set of photo-effect values.

Interpretation
The results are interpreted using the following table:

PIF < 2 or MPE < 0.1	No phototoxic
2 ≤ PIF ≤ 5 or 0.1 ≤ MPE ≤ 0.15	Probably phototoxic
PIF > 5 or MPE > 0.15	Phototoxic

If it is not possible to calculate an IC50 with or without UVA, this means that no PIF was determined for the test item. In this case, the value of the MPE will allow to conclude on the test item phototoxicity.

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RESULTS

Test validation
The negative control shows an absorbance higher or equal than 0.4.
The chlorpromazine, positive control, shows an IC_{50} comprise between 0.1 to 2 µg/mL when irradiated and 7 to 90 µg/mL when non-irradiated (PIF higher or equal than 6).
These results allow 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%.
The MPE is 0.058

CONCLUSION

Under the retained experimental conditions, the IC_{50} (-UV) and the IC_{50} (+UV) are not reached and the PIF cannot be calculated.
However, the COLIPA "phototox" software has found a MPE to 0.058.
The test item, CAPIXYL™ **tested at 15%** can be assigned as «non-phototoxic».

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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 CAPIXYL™ 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 CAPIXYL™ in reaction medium is 2 mg/L.
The Chemical Oxygen Demand is evaluated and equal to 0.95 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 CAPIXYL™ reaches **100.00**% after 28 days.

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, CAPIXYL™ **meets the easy biodegradability criteria at a concentration value of 2 mg/L in the reaction medium**. Therefore, CAPIXYL™ is **easily biodegradable**.

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DAPHNIA SP. ACUTE IMMOBILIZATION TEST

Study performed by Centre de Transfert de Technologie ODESSOL, 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 CAPIXYL™ 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 EC50 at 48h.
EC50 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 magna* is used for this assay.
CAPIXYL™ was tested at 5 concentrations: 0,01 mg/L; 0,1 mg/L; 1 mg/L; 10 mg/L and 100 mg/L (limit test)

Methods:
Daphnids (*Daphnia magna*), not older than 24 hours were exposed to 5 concentrations of CAPIXYL™ in 4 replicates each 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 during the test was adjusted to 21°C. The beakers were subjected to a light cycle of 16 hours with light intensity of 6000 lux followed by a dark cycle of 8 hours and these light/dark cycles lasted 48 hours.

Data are analyzed by an appropriate statistical method (e.g. probit analysis, etc.) to calculate the slopes of the curve and the EC50 with 95% confidence limit (p = 0.95).

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RESULTS

Mortality or immobility of the control: 0%

CAPIXYL™ conc. (mg/L)	Efficacy %
0,01	0
0,1	0
1	1,25
10	1,25
100	1,25

(EC50, 48h) of CAPIXYL™ is estimated to be equal to **557,62 mg/L.**

CONCLUSION

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

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CONCLUSION

Tolerance and safety studies have shown that CAPIXYL™ **at recommended usage level (5% and below)** presents no risk for cutaneous and/or ocular irritation. CAPIXYL™ has no skin sensitizing potential and is not mutagenic.

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

In conclusion to these toxicological studies, CAPIXYL™ is considered as well tolerated and totally safe for cosmetic purpose.

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