SCCNFP/0736/03

OPINION OF THE SCIENTIFIC COMMITTEE ON COSMETIC PRODUCTS AND NON-FOOD PRODUCTS INTENDED FOR CONSUMERS

CONCERNING

BASIC ORANGE 31

adopted by the SCCNFP during the 25th plenary meeting
of 20 October 2003

1. Terms of Reference

1.1. Context of the question

The adaptation to technical progress of the Annexes to Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products.

1.2. Request to the SCCNFP

The SCCNFP is requested to answer the following questions:

* Is Basic Orange 31 safe for use in cosmetic products?

* Does the SCCNFP propose any restrictions or conditions for its use in cosmetic products?

1.3. Statement on the toxicological evaluation

The SCCNFP is the scientific advisory body to the European Commission in matters of consumer protection with respect to cosmetics and non-food products intended for consumers.

The Commission's general policy regarding research on animals supports the development of alternative methods to replace or to reduce animal testing when possible. In this context, the SCCNFP has a specific working group on alternatives to animal testing which, in co-operation with other Commission services such as ECVAM (European Centre for Validation of Alternative Methods), evaluates these methods.
The extent to which these validated methods are applicable to cosmetic products and its ingredients is a matter of the SCCNFP.

SCCNFP opinions include evaluations of experiments using laboratory animals; such tests are conducted in accordance with all legal provisions and preferably under chemical law regulations. Only in cases where no alternative method is available will such tests be evaluated and the resulting data accepted, in order to meet the fundamental requirements of the protection of consumer health.

2. Toxicological Evaluation and Characterisation

2.1. General

2.1.1. Primary name

Basic Orange 31 (INCI)

2.1.2. Chemical names

Chemical name : 2-[(4-aminophenyl)azo]-1,3-dimethyl-1H-Imidazolium chloride
CAS name : 1H-Imidazolium, 2-[(4-aminophenyl)azo]-1,3-dimethyl-, chloride
Synonyms : /

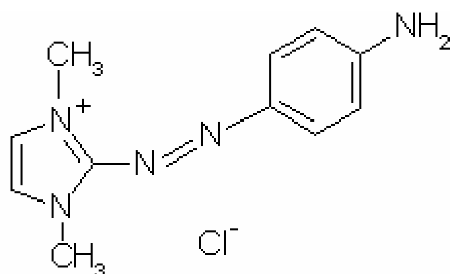
2.1.3. Trade names and abbreviations

Trade name : MIP Orange 3100, Vibracolor Flame Orange

2.1.4. CAS No. / EINECS No.

CAS No. : 97404-02-9
EINECS : 306-764-4

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_{11}H_{14}N_5.Cl$
Mol weight : 251.72

2.1.7. Purity, composition, and substance codes

Purity
Titre as determined by HPLC : 51.0 - 98.8 %

Water content : ≤ 1.9 %

Heavy metals	:	/
Potential impurities	:	≤ 0.3 % coloured by-product 1.3% MIP Red 2984 (batch no. 12R-10)
Salts of formulation or counter ions	:	≤ 45 % Sodium chloride ≤ 3.6 % Methyl sulfate ≤ 2.2 % sulfate
Reagents and intermediate reaction products	:	/
Solvent residues	:	/

2.1.8. Physical properties

Appearance	:	Violet/brown or dark red powder
Melting point	:	> 400 °C
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	-2.13, OECD Method No. 107 (1981)

2.1.9. Solubility

Water : 27.5 g/l at 20°C, OECD Method No. 105 (1995)

2.1.10 Stability

The test material has been reported to be stable in feed for at least 14 days at 20°C.

General comments on analytical and physico-chemical characterisation

* Purity of the dye in 4 batches varied from 51-98 %. A corresponding increase in the sodium chloride content was noted.
* Dye product of batch no. 12R-10 contained 1.3% MIP Red 2984. The chemical identification of this compound is not provided.
* No data on stability of the test material in feed up to 13 weeks has been provided. Also, there is no information on the stability of the test material in test solutions and hair dye formulations.
* The dye is an azo compound. It may release p-phenylenediamine. The content of p-phenylenediamine in the dye product should have been checked,

2.2. Function and uses

Basic Orange 31will be incorporated in non-oxidative hair dye formulations at a maximum concentration of 0.2%, and in oxidative hair dye formulations at a maximum final concentration of 0.1%, after mixing with the oxidative agent.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	/
Species/strain	:	Sprague Dawley Rat, Crl : CD (SD)IGS BR
Group Size	:	2 rats/sex per dose
Test material	:	MIP Orange 3100
Batch no	:	013673A1
Purity	:	94.1%
Dose	:	500, 1000, 2000 mg/kg bw
Observ. period	:	14 days
GLP	:	in compliance

In a dose-limit test, the test substance was dissolved in cell culture grade water and administered by gavage as single doses (mg/kg body weight) of MIP Orange 3100. One female from the mid and high dose groups died on Day 1 of the experiment. Clinical observations observed prior to death included salivation, lacrimation and hypoactivity. Macroscopic findings noted as red discoloured mucosal surface of stomach and distended organ filled with red lumen fluid in the stomach, caecum, ileum, duodenum, jejunum and colon.
All other animals survived until study termination; none showed signs of toxicity or adverse effects. The study indicated a LD_{50} between l000 mg/kg and 2000 mg/kg.

Ref. : 1

2.3.2. Acute dermal toxicity

Guideline	:	OECD Guideline 402
Species/strain	:	Sprague Dawley Rat, Crl : CD (SD)IGS BR
Group Size	:	5 male and 5 female
Test material	:	MIP Orange 3100
Batch no	:	013673A1
Purity	:	94.1 %
Dose	:	2000 mg/kg bw
Observ. period	:	14 days
GLP	:	in compliance

The test material was moistened with distilled water and applied at a dose of 2000 mg/kg bw. The hair was clipped the day prior to the experiment. It was applied to the clipped area as a thin uniform layer to approximately 10% of the body surface area from scapula to iliac crest and half way down the flank on each side of the animal's back. The area was occluded for 24 h. The initial dermal irritation was scored and recorded 30 minutes after bandage removal on Day 1. The untreated skin of each animal served as the control. Additional dermal irritation readings were performed for each animal on Days 3, 7, 10, and 14. All animals were examined for clinical signs of ill health or mortality immediately post-dose and approximately 1, 2.5 and 4 hours postdose, and daily thereafter. Body weights were recorded pre-dose on the day of dosing (Day 0), and on Days 7 and 14, and prior to sacrifice on Day 15 (fasted). A curtailed gross examination of the cervical, thoracic, and abdominal viscera was performed.

No deaths occurred during the study. All animals showed clinical signs of toxicity including chromodacryorrhea and red nasal discharge on the day of dosing and observation Day 1. All signs of toxicity were resolved at day 2. Body weight gain was not affected during the study and necropsy did not reveal observable changes. No irritation was noted throughout the study The acute dermal LD_{50} is greater than 2000 mg/kg bodyweight.

Ref. : 2

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

Guideline	:	OECD Guideline 407
Species/strain	:	Wistar Hanlbm (SPF) rat
Group Size	:	10 rats/sex : control and the high dose, 5 rats/sex: mid and low doses,
Test material	:	MIP ORANGE 3100
Batch no	:	CGF-F020088/0010
Purity	:	98%
Dose	:	20, 70 and 250 mg/kg bw
Treatment Period	:	14 days
Observ period	:	14 days
GLP	:	in compliance

MIP ORANGE 3100 was administered in feed at theoretical dose levels of 20, 70 and 250 mg/kg body weight/day while the control group received the normal diet. The corresponding effective daily intake for males and females was 15.5 mg/kg, 53.4 mg/kg and 186.4 mg/kg body weight, for the low, mid and high dose groups respectively. At the end of the 14 day treatment period, 5 animals of each dose and sex were killed. The remaining control and high-dose animals had a 14-day recovery period before sacrifice. The animals were examined for clinical signs daily and checked twice daily for mortality/viability. Food consumption and body weight were recorded once pre-test, and weekly thereafter and body weight at necropsy. A functional observational battery (modified Irwin screen test), grip strength and locomotor activity were performed during week 2. Blood samples for haematology and clinical biochemistry were collected from all animals, and urine samples were collected for analysis. All animals were killed and descriptions of all macroscopic abnormalities were recorded. The major organ weights (absolute and relative) were recorded on the date of necropsy. Samples of major organs from control and top dose groups, as well as liver and thyroid glands and all gross lesions from all animals were examined by light microscopy. Only liver, thyroid gland and gross lesions were examined microscopically from rats of mid and low dose groups.

All animals survived during the study. There were no quantitative or qualitative differences of the clinical parameters compared with the controls. Slight orange discoloration of the urine was observed in all dosed animals and was attributed to ingestion of the dye rather than to a specific toxic effect. Dose-related decreased food consumption was observed in the mid and high dose group males and in the high-dose group females. The relative food consumption reflected these differences. Mean body weight and body weight gain were lower in high-dose group compared with the control. The functional observational battery tests, grip strength and locomotor activity were similar in the high dose and control groups.

The observed haematological effects (slightly prolonged activated partial thromboplastin time), clinical biochemical changes (increase of triglyceride levels in males and elevated albumin and total protein level in females at the high-dose group) or urinalysis parameters (pH slightly more alkaline in females at the high-dose) were considered to be incidental and unrelated to the treatment. No test substance-related differences in any organ weights or organ weight ratios compared with the controls were evident either at the end of the treatment or recovery. All macroscopic and microscopic findings observed (renal pelvic dilatation, bilateral dilatation of the uterine horns, incomplete deflation of the lung, one renal nephroblastoma) were considered to be within the normal range of background findings commonly seen in rats of this strain and age and not related to MIP Orange 3100.

These results suggest a NOEL of MIP Orange 3100 at 15 mg/kg body weight/day and a NOAEL of 53 mg/kg bw.

Ref : 4

2.3.5. Repeated dose dermal toxicity

Guideline	:	OECD Guideline 402 (1987)
Species/strain	:	Albino Guinea pig
Group Size	:	4 males and 4 females
Test material	:	MIP ORANGE 3100
Batch no	:	CGF-F020088/0010
Purity	:	98%
Dose	:	5.0, 3.0, 1.0 or 0.5 w/w
Observ. period	:	14 days
GLP	:	in compliance

To assess the cumulative irritation potential, MIP ORANGE 3100 was applied daily at concentrations of 5.0, 3.0, 1.0 or 0.5 % w/w in double distilled water. One male and female served as controls and received only double distilled water. Two of the four different concentrations (5.0, 3.0, 1.0 or 0.5 % w/w) were applied daily. Each application was 0.1 ml and was made to separate 7 cm^2 shaved areas on the back of each of the treated animals. The control or dose sites were not occluded. Each day of treatment each concentration was applied three times, each time on three different animals. This was repeated for 14 daily treatments.

Due to slight accumulation of the test substance, no grading scores were possible from Day 2 –14, as no depilation was used during this period. After depilation, the MIP Orange 3100 application sites remained coloured.
Macroscopic isolated reddish foci in the subcutis were noted in one animal at 1 % dose. The skin, at vehicle alone sites, showed no histopathological changes. The MIP Orange 3100 application sites showed minimal to moderate epidermal hyperplasia and in some cases with minimal to slight dermal inflammatory cell infiltrate. These observations were not dose-related. No deaths occurred during the study.

MIP Orange 3100 is considered to be non-irritant when tested under these experimental conditions. A NOEL could not be established under these experimental conditions.

Ref : 3

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline : OECD 408 (1981) and Directive 96/54/EEC
Species/strain : Wistar rat, Hannover (SPF)
Group Size : 10 males + 10 females per dose
Test material : MIP ORANGE 3100
Batch no : CGF-F020088//0010
Purity : 98%
Dose : 0, 20, 70 and 250 mg/kg bw/day
Exposure period : 13 weeks
GLP : in compliance

MIP Orange 3100 was administered in feed at theoretical dose levels of 20, 70 and 250 mg/kg body weight/day while the control group received the normal diet. The corresponding effective daily intake, based on food consumption and body weight for males was 18, 63 and 229 mg/kg body weight, and for females 19, 66 and 232 mg/kg bw for low, mid and high dose groups respectively.

The animals were examined for clinical signs daily and checked twice daily for mortality/viability. Food consumption and body weight were recorded once pre-test, and weekly thereafter and body weight at necropsy. Ophthalmoscopic examination was performed at pre-test and at week 13 (control and high-dose animals). A functional observational battery (modified Irwin screen test) was performed during pre-test and at week 12 on all rats and grip strength and locomotor activity were evaluated. At week 13, blood and urine were analysed. After 13 weeks, all animals were weighed and killed and descriptions of all macroscopic abnormalities were recorded. The major tissues and organ were collected from all animals and absolute and relative weights were recorded at necropsy for adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thyroid, and thymus. Samples of major organs from control and high-dose and all gross lesions from all animals were processed as haematoxylin-eosin slides and examined by light microscopy.

No deaths occurred during the study. In the high dose group, there was a 17% reduction in mean food consumption in males and 8% in females. These data were considered test substance-related. The only clinical signs related to the test substance were in the high dose group. These were orange urine from Day 3 and orange faeces from Week 4 and a reduction in food consumption (17% in males, 8% in females). This resulted in significant decreased body weight and body weight gain in males ($p < 0.05$). No treatment-related effects in the functional observational battery, grip strength measurement, and locomotor activity were noted.

Methaemoglobin levels were statistically significant increased in the high-dose group. Also in the high-dose group, there was a significant ($p > 0.05$ or $p > 0.01$) reduction including glucose and urea in the males, creatinine in both males and females, and alpha-1 globulin in females. There were also significant ($p > 0.05$ or $p > 0.01$) increases of total cholesterol, triglycerides, phospholipids, albumin/globulin ratio, and gamma glutamyltransferase. These findings did not have microscopically observable adverse effects so they are considered to be adaptive responses to the test article and of no toxicological relevance. In the mid- and low-dose groups none of the small number of clinical chemistry changes were considered to be adverse effects of the test substance. Urinalysis did not reveal any differences from control.

Compared with the control group, a slight increase in relative kidney weight and a decrease in relative heart weight were observed and attributed to the test article at the high dose of 250 mg/kg. All macroscopic findings observed were similar in treated and control animals. Some microscopic

findings, classified as either minimal or slight, differed from treated and controls rats: hepatocellular hypertrophy considered as a metabolic response to the treatment.

The study authors estimated the NOEL to be 18 mg/kg body weight/day and the NOAEL, 60 mg/kg. bw/day.

Ref : 5

2.3.8. Sub-chronic dermal toxicity

No data

2.3.9. Sub-chronic inhalation toxicity

No data

2.3.10. Chronic toxicity

No data

2.4. Irritation and corrosivity

2.4.1. Irritation (skin)

Guideline : OECD 404 (1981)
Species/strain : albino rabbits, New Zealand
Group size : 2 males, 1 female
Test substance : MIP Orange 3100
Batch number : 013673A1
Purity : 94.1% (expressed as the chloride)
Dose : 0.5g applied to 6.25 cm^2 of intact skin for 4 hours
GLP : In compliance

After clipping the back and flanks, 0.5g of the test material, moistened with approximately 0.5ml distilled water, was applied to an area of approximately 6.25 cm^2 under a semi-occlusive dressing. The patches were removed after 4 hours, residual test article wiped off, and observations made at 0.5, 1, 24, 48 and 72 hours after removal.

Results
No erythema was observed. Very slight oedema was noted in one animal at the 0.5 to 1-hour observation. The primary irritation index was 0.1. The test material was considered to be *slightly irritating* to rabbit skin under the conditions of the study.

Ref. : 6

2.4.2. Irritation (mucous membranes)

Study 1

Guideline : OECD 405 (1981)
Species/strain : albino rabbits, New Zealand

Group size	:	3 females
Test substance	:	MIP 3100
Batch number	:	013673A1
Purity	:	94.1% (expressed as the chloride)
Dose	:	0.045 g (neat substance)
GLP	:	In compliance

The test material was placed into the everted lower lid of the right eye of each animal. The left eye served as the untreated control. The eyes of the 3 animals remained unrinsed for approximately 24 hours after instillation of the test material.

1, 24, 48, 72 and 96 hours and 7, 14 and 21 days after instillation of the test material, the treated eyes of the rabbits were observed for signs of ocular irritation. Corneal injury was assessed using sodium fluorescein (followed by a saline wash) on all animals at 24 hours post-instillation.

Results
Changes to the cornea, iris and conjunctivae were noted in all three animals. On day 5, two animals were sacrificed due to severe ocular irritation. The third animal had a positive sodium fluorescein test at 24 and 72 hours and a negative test at 96 hours. The findings for this animal began to resolve on day 7 through day 21 postinstillation.
MIP 3100 was severely irritating to the eye under the test conditions of study 1.

Ref. : 7

Study 2

Guideline	:	OECD 405 (1981)
Species/strain	:	albino rabbits, New Zealand
Group size	:	3 females
Test substance	:	MIP 3100
Batch number	:	013673A1
Purity	:	94.1% (expressed as the chloride)
Dose	:	0.1ml of 1% w/v
GLP	:	In compliance

The test material was instilled into the everted lower lid of the right eye of each animal. The left eye served as the untreated control. The eyes of the 3 animals remained unrinsed for approximately 24 hours after instillation of the test material.

1, 24, 48, 72 and 96 hours and 7, 14 and 21 days after instillation of the test material, the treated eyes of the rabbits were observed for signs of ocular irritation. Corneal injury was assessed using sodium fluorescein (followed by a saline wash) on all animals at 24 hours post-instillation.

Results
There were no findings involving the cornea or iris noted in any animal. All three animals showed conjunctival redness (score 1) at 1-hour and 24-hours postinstillation and discharge (score 1) in one animal at 1 hour postinstillation.

A 1% solution of MIP 3100 was slightly irritating to the eye under the test conditions of study 2.

Ref. : 7

2.5. Sensitisation

Magnusson and Kligman Guinea pig maximisation test

Guideline	:	OECD 406		
Species/strain	:	albino guinea pigs		
Group size	:	30 females (20 test and 10 control)		
Test substance	:	MIP 3100		
Batch number	:	MIP 3100/23 R-1		
Purity	:	56.1 % of base (with 19.3% sodium and 25.7% chloride)		
Dose	:	Intradermal induction	:	5% aqueous solution with Freund's Complete Adjuvant.
		Topical induction	:	A 50% dilution of test material under occlusion for 48 hours. Controls received vehicle only. Skin pretreated with 10% sodium lauryl sulphate in liquid paraffin.
		Challenge	:	Performed on day 22 (14 days epidermal applications) by exposing 50% aqueous dilution of the test substance (24 hours, occlusion).
GLP	:	In compliance		

Animals were examined 24 and 48 hours after removal of the patches for signs of erythema and oedema.

Results
Brown-red discoloration was noted directly after removal of patches. To remove discoloration, all animals were depilated 3 hours prior to challenge reading with a depilatory cream (VEET). None of the animals of the control or test group were observed with skin reactions after challenge with a non-irritating dilution of 50 % of the test material. MIP 3100 was considered not to be a sensitiser under the test conditions.

Ref. : 9

Local lymph node assay

Guideline	:	OECD 429
Species/strain	:	CBA/J mice
Group size	:	28 females (7 groups of 4 animals)
Test substance	:	MIP 3100
Batch number	:	013673A1
Purity	:	94.1%
Dose	:	0.25, 0.5, 1, 2.5 and 5%
GLP	:	In compliance

Five treated groups received MIP 3100 at concentrations of 0.25, 0.5, 1, 2.5 and 5%. A negative control received the vehicle (ethanol/water – 50/50 v/v). A positive control group received 25% alpha-hexylcinnamaldehyde.
The test items were applied over the ears (25µL per ear) for three consecutive days. After 2 days of resting, the proliferation of the lymph node cells in the lymph node draining the application

site was measured by incorporation of tritiated-methyl thymidine. The obtained values were used to calculate stimulation indices (SI).
The irritant potential of the test item was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
No increase in ear thickness was observed in the animals of the treated group. A red coloration of the skin, which could have masked an erythema, was noted on the ears of the treated animals from day 2 up to the end of the study.

A dose-related increase in the stimulation index was noted and the threshold positive value of 3 was exceeded at the concentrations of 1 and 5%.

In the absence of local irritation, the positive lymphoproliferative responses observed at 1 and 5% were attributed to delayed contact hypersensitivity.
The extrapolated EC3 value for the test item MIP 3100 was 3.12%.

MIP 3100 induces delayed contact hypersensitivity in the murine Local Lymph Node Assay.
Ref. : 8

2.6. Teratogenicity

Guideline	:	CEC, N° 111/3387/93, according to ICH guidelines.
Species/strain	:	Wistar rat, Hanlbm (SPF)
Group Size	:	22 mated females per dose
Test material	:	MIP ORANGE 3100
Batch no	:	CGF-F020088//0010
Purity	:	98%
Dose	:	0, 15, 60 and 240 mg/kg bw/day
Treatment period	:	Days 6 to 17 post coitum,
GLP	:	in compliance

Groups of 22 mated female rats were dosed with 10ml /kg aqueous solution of MIP Orange 3100 by gavage once daily. The control group received only the vehicle (double distilled water). Food consumption was recorded for the following periods: days 0-6, 6-12, 12-18 and 18-21 post coitum; body weight was recorded daily from day 0 until day 21 post coitum. Clinical observations and deaths were recorded at least twice daily. After sacrifice on day 21 post coitum, all internal organs were examined, with emphasis on the uterus, uterine contents, position of foetuses in the uterus and number of corpora lutea. The uteri of all females with live foetuses were weighed at necropsy; the foetuses were removed from the uterus, weighed, and examined for sex and gross external abnormalities.
There were no deaths of dams during the treatment period. Reduced food consumption (-8.6% and -20.9% in the mid-and high dose groups) was observed and reflected in diminished body weight gain. The bedding was stained orange, assumed to be due to excretion in the urine and faeces of parent compound or metabolites.

Mean post-implantation loss and mean number of foetuses per dam were similar between treated and control dams. There was a slight decrease in mean foetal body weights in the high-dose group. Statistically significant differences were observed in the sex ratio of foetuses (fewer male to female foetuses) in the mid- and high dose groups. The study authors considered the difference not

treatment related since implantation occurred prior to dosing, litter size and post-implantation losses were unaffected. Some abnormal findings were noted on external features (one abdominal hernia in the low-dose group; low weight, cleft palate and tail defects in the high-dose group) and skeletal parameters (small number of shaped sternebrae or wavy ribs in all groups, delayed ossification in the high dose group). Delayed ossification commonly occurs secondary to maternal toxicity. Thus the skeletal findings were considered unrelated to the tested product.

Based on these results, the study authors considered the NOAEL to be 60 mg/kg body weight/day for maternal and foetal effects. MIP Orange 3100 did not reveal any teratogenic effects.

Ref. : 12

2.7. Toxicokinetics (including Percutaneous Absorption)

In vitro study of percutaneous absorption

Guideline	:	/
Tissue	:	Human epidermal skin membrane (exposure area: 1 cm²)
Method	:	Franz diffusion cells
Test material	:	Basic Orange 31, 0.200 % dye in a hair dye formulation
Batch No	:	CGF-F020088/0010, purity: 98.0 %
Dose level	:	101 mg/cm² of the formulation; 202 µg/cm² of the dye active principle
Receptor fluid	:	25% (w/v) ethanol in PBS saline (pH 7.4)
Replicate cells	:	12
Analytical method	:	HPLC (detection at 486 nm). Detection limit: about 1 ng/ml.
GLP	:	In compliance

The skin penetration of Basic Orange 31 was evaluated in a static Franz diffusion cell system using epidermal membranes obtained from full-thickness human female skin. The integrity of the skin was checked by $[^3H]$-H_2O flux (limit of permeability coefficient: 1.5 * 10^{-3} cm/h. The solubility of the dye in the receptor fluid was higher than 200 µg/ml.
The dye formulation (101 mg/cm²) equivalent to 202 µg/cm² of the dye active principle was applied on the skin surface for 30 min. Then, the skin surface excess was washed off with warm water (40 ºC) and left unocluded for the entire 48 hour exposure period. At the end of the experiment, the dye content was determined in the following compartments: skin surface excess (including washings, wipes and donor chamber), SC, epidermis and receptor fluid.

Results
Under the present experimental conditions, a total recovery of the dye of 99.0 % has been obtained. Most of the hair dye applied on the skin surface was removed with the washing procedure (98.95 % or 200.1 µg/cm²). The content of the dye detected in the different strips of the SC was: 0.017 % (0.039 µg/cm²). A total of 0.009 % of the applied dose is reported to have penetrated into the epidermis (0.004 %) and permeated into the receptor fluid (0.005 %) during 48 h. This corresponds to a percutaneous absorption of 0.018 µg/cm².

Comment
The substance was not tested in the presence of an oxidising agent. The applied dose of 101 mg/cm² is higher than the amount recommended by the SCCNFP (20 mg/cm²).

Ref. : 13

2.8. Mutagenicity/Genotoxicity

2.8.1. Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471
Species/strain	:	*S. typhimurium,* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	Basic Orange 31 in water
Batch no	:	013673A1, purity : 94.1 %
Concentrations	:	Test #1 *Salmonella typhimurium* Without metabolic activation 10.0, 33.3, 100, 333, 1000, 2000 µg/plate (6 doses) *E. coli* Without metabolic activation 3.33, 10.0, 33.3, 100, 333, 500 µg/plate (6 doses) *Salmonella typhimurium and E. coli* With metabolic activation (rat liver) 33.3, 100, 333, 1000, 2000, 3330 µg/plate (6 doses) With reductive metabolic activation (hamster liver) 33.3, 100, 333, 1000, 2000, 3330 µg/plate (6 doses) Test #2 Without metabolic activation 33.3, 100, 333, 1000, 2000, 3330 µg/plate (6 doses) With metabolic activation (rat liver) 33.3, 100, 333, 500, 667, 1000 µg/plate (6 doses) With reductive metabolic activation (hamster liver) 33.3, 100, 333, 667, 1000, 2000 µg/plate (6 doses)
GLP	:	In compliance

Basic Orange 31 has been investigated for gene mutation in *S. typhimurium and E. coli* using the preincubation method both with or without S9 mix. In the confirmatory assay standard and reductive exogenous activation systems.
S9 mix from different origin have been used : Standard : Sprague-Dawley rats injected i.p. with Aroclor™ 1254 ; Reductive : uninduced male Golden Syrian hamsters.
Negative and positive controls were in accordance with the OECD guidelines.

Results
Dose range finding assay
Inhibition of growth, as evidenced by a decrease of revertant frequency or thinning of the background lawn was observed in the tester strains

TA 100	at doses ≥ 333 µg/plate in the presence of S9 and; at doses ≥ 33.3 µg/plate in the absence of S9.
E. coli	at doses ≥ 333 µg/plate in the presence of S9 and; at doses ≥ 333 µg/plate in the absence of S9

Test # 1 (initial)
* In the absence of activation : no dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains (*Salmonella or E. coli*).
* In the presence of rat (commonly used S9) activation : No dose related and biologically relevant increase in revertant numbers was observed, in almost any of the tester strains used (*Salmonella or E. coli*).
However, for the tester strain TA 98 at the dose of 100 µg/plate, individual plate counts were as follow (ctrl : 46, 47 ,42 / 100 µg : 96, 123, 99). The mean value is therefore 45 ± 3 for the Ctrl and 106 ± 15 for the 100 µg/plate. This increase is not mentioned in the main text and its biological relevance not commented while being positive according to the positivity criteria (2 times the ctrl value for TA 98).
In the presence of Hamster (reductive S9) activation : an increase in revertant numbers was observed for TA 98 - a frameshift tester strain – from the lowest dose of 33.3 µg/plate. The increases observed are dose-dependent up to doses that induce cytotoxicity, they should be considered as biologically relevant. The maximal mean value observed is approximately 7.2 times the mean revertant control values.
For the other strains, no statistically or biologically relevant increase of mutant frequencies have been observed as compared to the controls.
Positive controls showed the expected response.

Test # 2 (confirmatory)
In the absence of activation : No dose related and biologically relevant increase in revertant numbers was observed, in any of the tester strains.
This absence of a dose-related increase in revertant numbers is also observed in almost any of the tester strains used (*Salmonella or E. coli*) in the presence of rat (commonly used S9) activation.
A similar finding as in experiment # 1 was observed, for the tester strain TA 98 at the dose of 100 µg/plate, individual plate counts were as follow (ctrl : 27, 28 ,25 / 100 µg : 72, 87, 95). The mean value is therefore 27 ± 2 for the Ctrl and 85 ± 12 for the 100 µg/plate. This increase is not mentioned in the main text and its biological relevance not commented while being positive according to the positivity criteria (x 2 the ctrl value for TA 98).
In the presence of Hamster (reductive S9) activation : an increase in revertant numbers was observed for TA 98 - a frameshift tester strain –from the lowest dose of 33.3 µg/plate. The increases observed are dose-dependent up to doses that induce cytotoxicity, they should be considered as biologically relevant. The maximal mean value observed is approximately 15.5 times the mean revertant control values. For the other strains, no statistically or biologically relevant increase of mutant frequencies have been observed as compared to the controls.

Conclusions
The test is acceptable for evaluation.
Based on the reversion rate, and under the conditions of the 2 assays performed, it may be concluded that the test agent Basic Orange 31, in the presence of reductive S9 mix, shows clear evidence of mutagenic activity in tester strain TA 98. Such positive results may be the consequence of the metabolizing properties (azo-reduction) of the S9 mix fraction from hamster. The higher amount of aromatic amines released, which are metabolised to electrophilic molecules, may react with DNA. This might explain the positive results observed in TA 98.
Ref. : 14

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline : OECD 476
Cells : Chinese Hamster V-79 cell line (mutation at the HPRT locus)
Replicates : 2 independent tests
Test substance : Basic Orange 31 in water
Batch no : 12R-10
Purity : 98.8 %
GLP : In compliance

Concentrations : Test #1
Without metabolic activation :
3.0, 10.0, 30.0, 100.0, 300.0, 600.0 µg/ml (6 doses)
With metabolic activation (Reductive hamster S9 mix) :
3.0, 10.0*, 30.0, 100.0, 300.0, 600.0** (6 doses)

Test #2
Without metabolic activation :
3.0, 10.0*, 30.0, 100.0*, 300.0, 400.0 µg/ml (6 doses)
With metabolic activation (Rat S9 mix) :
3.0, 10.0*, 30.0, 100.0, 300.0, 400.0** µg/ml (6 doses)

* : culture not evaluated
** : no evaluation due to strong toxicity.

Basic Orange 31 has been investigated for gene mutation at the HPRT locus in V79 chinese hamster cell line in the presence or absence of activation system. No visible precipitate occurred. In the first experiment, S9 activation system was derived from Hamsters while liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system in the second test.
Results
Test # 1
Mutant frequencies in the absence or presence of activation.
No statistically or biologically significant increase in mutant frequency with no dose-related increase was observed over the concurrent solvent controls for any doses.
(ctrl 6.2 per million cells/ - S9 ; ctrl 0.5 per million cells/ + S9)
(exposed : with hamster S9 mix : 10.8, 10.1, 4.6, 13.5).
(exposed : without S9 mix 0.6, 16.8, 9.0, 20.1)

Test # 2
Mutant frequencies in the absence or presence of activation.
No statistically or biologically significant increase in mutant frequency with no dose-related increase was observed over the concurrent solvent controls for any doses.
(ctrl 4.4 per million cells/ without S9 mix ; ctrl 3.0 per million cells/ with S9 mix)
(exposed : with rat S9 mix : 1.8, 5.6, 5.0, 18.9).
(exposed : without S9 mix 01.4, 3.9, 5.0, 10.4)

Conclusions

No statistically or biologically relevant significant increase in mutant frequency was observed over the concurrent solvent controls after treatment with Basic Orange 31 in either test in the presence of rats or hamster S9 activation or absence of activation.
Therefore, the test substance Basic Orange 31 does not demonstrate mutagenic potential on the HPRT gene of V79 cells.
Remark : it should be noted that the test agent expresses a clear cytotoxic effect.

Ref. : 16

***In Vitro* Mammalian Chromosomal Aberration Test**

Guideline	:	OECD 473
Species/strain	:	Human lymphocytes (from 2 healthy donors, pooling not described)
Replicates	:	Duplicate cultures, 2 independent experiments
Test substance	:	Basic Orange 31 in water
Batch no	:	013673A1
Purity	:	94.1 %
Concentrations	:	Test #1 without S9 mix 3 h treatment – 22.1 h harvest : 33.7, 48.1, 68.7, 98.1 µg/ml with S9 mix 3 h treatment – 22.1 h harvest : 98.1, 140.0, 200.0, 285.0 µg/ml Test # 2 without S9 mix 22 h treatment – 22 h harvest : 3.13, 6.25, 12.5, 25.0 µg/ml with S9 mix 3 h treatment – 22 h harvest : 25.0, 50.0, 100.0, 200.0 µg/ml
GLP	:	In compliance

Basic Orange 31 has been investigated for induction of chromosomal aberrations in human lymphocytes withdrawn from 2 volunteers. The test concentrations were established from a preliminary toxicity study.
Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system.

Results
pH & Osmolarity
At the maximum dose tested of 4570 µg/ml, the pH was not significantly changed (pH =7.5)

Structural chromosome aberrations
Experiment # 1, without S9 mix
No statistically or biologically significant increase in the number of aberrant cells was observed as compared to the corresponding solvent control for all doses except the top one. At this concentration (98.1 µg/ml) a mean value of 6 % of aberrant cells were observed as compared to 0.5 % in the control group. Qualitatively speaking, the aberrations described were mostly simple breaks : mean 5.5 % and chromatid exchanges : mean 1.0 %. However, such aberrations occurred at a concentration that produced more than 50 % of MI reduction. This increased is only observed at one concentration and its biological significance questionable.
No significant increase of aneuploidy and/or endoreduplicated cells was noted.

Experiment # 1, with S9 mix (from induced rats)
No statistically or biologically significant increase in the number of aberrant cells was observed as compared to the corresponding solvent control for all doses.
No significant increase of aneuploidy and/or endoreduplicated cells was noted.

Experiment # 2, without S9 mix or with S9 mix (from induced rats)
No statistically or biologically significant increase in the number of aberrant cells was observed as compared to the corresponding solvent control for all doses.
No significant increase of aneuploidy and/or endoreduplicated cells was noted.
Conclusions
Basic Orange 31 is considered negative for its clastogenic and/or aneugenic potential in human lymphocytes in the presence or the absence of activation system under the conditions of the test. The test has only been performed with a standard metabolic activation system. No definitive conclusions can be made at present.

Ref. : 15

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474
Species	:	NMRI mice
Group sizes	:	6 male and 6 female
Material	:	Basic Orange 31 in deionized water
Batch no	:	CGF-F020088/0010
Purity	:	98 % (HPLC)
Dose levels	:	Maximum Tolerated Dose (MTD) Four preliminiminary dose-range finding assays were conducted. According to clinical signs and toxic reactions of the mice, the top dose has been chosen to be 300 mg/kg bw Basic Orange 31 was administered by 1 single oral dose of 30.0, 100.0 and 300 mg/kg bw for the 24 h sacrifice time 300 mg/kg bw for the 48 h sacrifice time.
GLP	:	In compliance

Basic Orange 31 has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by 4 preliminary range finding studies in which observable toxic effects were seen at doses of 1000 and 2000 mg/bw. The substance was administered by a single intragastric gavage and the groups of animals sacrificed 24 and 72 hours after administration. Negative and positive controls were in accordance with the OECD guideline.

Number of cells scored : a total of at least 2000 erythrocytes were examined from each animal; the incidence of micronucleated erythrocytes and the ratio of polychromatic erythrocytes to normochromatic erythrocytes were calculated.

Results
* NCE : the mean number of NCE (mature differentiated cells) was increased after treatment as compared to controls; Ctrl : 1588/ 24 h : 1559, 1611, 2001 ; 48 h : 2578) this indicates cytotoxicity of the test agent.

* µ PCE 24 h sampling time : while an increase with a trend for dose dependency was noted, its amplitude is small; no statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic erythrocytes over the concurrent vehicle control values were observed for any dose levels. (Ctrl : 0.85 per 1000; 30 mg/kg : 0.7 per 1000; 100 mg/kg : 0.95 per 1000; 300 mg/kg : 1.3per 1000). Cyclophosphamide 40 mg : 16.9 per 1000.

* µ PCE 48 h sampling time : no statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic erythrocytes over the concurrent vehicle control values were observed. (Ctrl : 0.85 per 1000; 300 mg/kg : 0.5 per 1000)

Remarks : for some doses it is mentioned : * mean of 2 independent scoring (different samples 2000 PCE per scoring. However, the raw data are not given. The rational for the separate scoring should be indicated.

Conclusions
Under the conditions of the test it can be concluded that Basic Orange 31 at doses at which some signs of clinical toxicity were recorded, does not induce statistically significant increase in the frequency of µ PCE.

Ref. : 18

Unscheduled DNA Synthesis (UDS) Test With Mammalian Liver Cells *in vivo*

Guideline	:	OECD draft guideline 486
Species/strain	:	Wistar rat, HanIbm: WIST (SPF) strain
Group size	:	4 males rats
Test substance	:	Basic Orange 31
Batch No	:	CGF-F020088/0010
Purity	:	98.0 %
Dose levels	:	Maximum Tolerated Dose (MTD) : 2 preliminary dose-range finding assays were conducted. The top dose of Basic Orange 31 was chosen on the basis of clinical signs and toxic reactions of the treated rats ; the top dose has been chosen to be 400 mg/kg bw.
	:	A single oral dose was given to a group of male rats at dose levels of 100 and 400 mg/kg. Two sampling times were selected : 3 h & 16 h post-treatment.
Exposure time	:	3 h and 16 hours: all dose groups
GLP	:	In compliance

Basic Orange 31 has been investigated for induction of unscheduled DNA synthesis in rats hepatocytes at 2 doses 100 and 400 mg/kg bw.
Positive controls are in accordance with OECD guideline and UDS analysed by autoradiography.
3 males were used per dose/time sampling

Results
* The viability of the hepatocytes was not substantially affected by the treatments
* Treatment with Basic Orange 31 at doses of 100 & 400 mg/kg yielded group mean NNG values less than 0 for both experiment time and caused no significant increases, as compared to control, in the mean nuclear grain counts.
* The percentage of cells in repair did not significantly differ from the control group.

Conclusions
Data indicate that single oral gavage treatment of male rats dosed once with 100 & 400 mg/kg of Basic Orange 31 did not induced increased unscheduled DNA synthesis in hepatocytes isolated approximately 3 or 16 hours after dosing.
Under the experimental conditions, it is concluded that Basic Orange 31 did not display DNA repair activities detectable by this assay.

Ref. : 17

2.9. Carcinogenicity

No data

2.10. Special investigations

Photoirritation

Guideline	:	OECD draft (1995) "Acute dermal photoirritation dose-response test"
Species/strain	:	Himalayan spotted guinea pigs
Group size	:	15 males (10 test and 5 control)
Test substance	:	MIP 3100
Batch number	:	CGF-F020088/0010
Purity	:	98%
Dose	:	0.025 ml/2cm^2 of 50%, 25%, 15% and 10% aqueous dilutions. Skin at test sites treated 30 minutes before application with 2% DMSO in ethanol.
GLP	:	In compliance

MIP 3100 was applied epicutaneously to skin areas of 2 cm^2 on both flanks. 30 minutes after application of the test materials, the left flank was exposed to 20 J/cm^2 UVA. The right flank remained unexposed to light after treatment and served as a reference site. Control animals were exposed to UVA similarly but treated with solvent only. Cutaneous reactions were evaluated at 24, 48 and 72 hours after application. Before the 24 hour reading, the application sites of the test animals were depilated (VEET cream).

Results
There was no reaction observed in any animal and it was concluded that under the test conditions, MIP 3100 does not exhibit a phototoxic potential in the guinea pigs of the strain and age.

Ref. : 10

Photoallergy

Guideline	:	CTFA Safety Testing Guideline
Species/strain	:	Himalayan spotted guinea pigs
Group size	:	30 males (20 test and 10 control)
Test substance	:	MIP 3100
Batch number	:	MIP 3100/48 R-9 (CGF-F020088/0010)
Purity	:	98%

Dose : Induction : nuchal skin of the test group shaved. Test site of 6-8 cm^2 defined by four 0.1ml intradermal injections of Freunds Complete Adjuvant and physiological saline 1:1 into the corners. 0.1ml of 50% MIP 3100 applied to area of 8 cm^2. The site was then exposed to 1.8 J/cm^2 UVB and $10J/cm^2$ UVA. The application and irradiation (after shaving) was repeated on days 3, 6, 8, and 10.

Challenge : 3 weeks after the start of the induction procedure, test sites of $2cm^2$ were marked and 0.025 $ml/2cm^2$ of 50%, 25%, 15% and 10% were applied to the left flank and then irradiated with $10J/cm^2$ UVA. After irradiation of the left flank, the right flank was treated with the test materials without irradiation.

GLP : In compliance

3 hours prior to the first readings, the application sites were depilated with a depilatory cream (VEET cream). Each animal was assessed for reactions at 24, 48 and 72 hours after challenge.

Results
A very slight red discoloration produced by the test material at the application sites were observed from test days 2 to 23. No skin reactions were observed in the test animals treated with 50% test material during the induction phase. No reactions were observed on the irradiated or non-irradiated flanks of the control and test animals treated with 50, 25, 15 and 10% test material.
The data indicates that MIP 3100 does not exhibit photoallergic potential.

Ref. : 11

2.11. Safety evaluation

Not applicable

2.12. Conclusions

Purity of the dye in different batches varied from 51% to 98%. A corresponding decrease in the sodium chloride content was noticed. Vacuum drying and recrystallisation will produce high purity dye with less salt content. A high salt content may increase dermal penetration of the dye. The chemical identification of 1.3% Basic Orange 31 in one of the test batches is not provided. Data provided on stability of the test material is insufficient.

Acute oral toxicity : none of the animals showed signs of toxicity or adverse effects. The study indicated a LD_{50} greater than l000 mg/kg but less than 2000 mg/kg. The acute dermal LD_{5O} is greater than 2000 mg/kg bw.
A repeated dose oral toxicity suggests a NOAEL of 53 mg/kg bw/day. The sub-chronic oral toxicity yielded a NOEL of 18 mg/kg bw/day and an NOAEL of 60 mg/kg bw/day.
Basic Orange 31 did not reveal any teratogenic effects. The NOAEL was set at 60 mg/kg bw/day for maternal and foetal effects.

The test material was considered to be slightly irritating to the skin. A 1% solution was slightly irritating to the eye.
It was considered not to be a sensitiser. It induces delayed contact hypersensitivity in the murine Local Lymph Node Assay.

Basic Orange 31 does not exhibit neither a phototoxic nor a photoallergic potential.

A total of 0.009 % of the applied dose is reported to have penetrated, corresponding to a percutaneous absorption of 0.018 µg/cm². However, the substance was not tested in the presence of an oxidising agent. The applied dose of 101 mg/cm² is higher than the amount recommended by the SCCNFP (20 mg/cm²).

Basic Orange 31 has been tested in prokaryotic and mammalian cells for gene mutation, and in mammalian cells for chromosomal aberration in vitro. Two in vivo tests have been performed (bone marrow micronucleus and UDS tests).
Considering that the metabolic pathways suspected in the strain TA 98, could have influenced specifically the results observed so far and therefore that the increase observed is devoid of biological significance, the in vitro test for gene mutation in prokaryotes has been found negative in all the others tester strains also in the presence of a reducing metabolic activation system.
The in vitro test for gene mutation in mammalian cells showed that the test agent is non mutagenic in the absence of activation system and under normal or reduced activation systems.
The *in vitro* test for clastogenicity in human lymphocytes is negative in the presence of a normal metabolic system.
The *in vivo* micronucleus test in mice gave negative results.
The *in vivo/in vitro* UDS on rat hepatocytes is negative for the treatment of 3 and 16 hours.

Derived from the available data, Basic Orange 31 is considered non mutagenic/genotoxic.

2.13. References

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3. Opinion of the SCCNFP

The SCCNFP is of the opinion that Basic Orange 31might be regarded as safe in general. However, the data were insufficient for a final evaluation.

Further information is required on the chemical identification of all impurities and data on the stability of the test material used in the experimental investigations and in hair dye formulations. A percutaneous absorption study in accordance with the Notes of Guidance is required.
The safety dossier should fulfil the demands of the SCCNFP "Strategy Paper" (doc. n° SCCNFP/0720/03 of 24.06.2003) as to mutagenicity of possible reaction products.

4. Other considerations

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5. Minority opinions

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