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

CONCERNING

HC YELLOW N° 9

COLIPA n° B69

adopted by the SCCNFP during the 24th plenary meeting
of 24-25 June 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 HC Yellow n° 9 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

HC Yellow No 9 (INCI name)

2.1.2. Chemical names

Chemical name : 1-methoxy-3-(β-aminoethyl)amino-4-nitrobenzene, hydrochloride
CAS name : 1,2-ethanediamine, N-(5-methoxy-2-nitrophenyl)-, monohydrochloride
Synonyms : N-(5-methoxy-2-nitrophenyl)-1,2-ethanediamine, monohydrochloride
N1-(5-methoxy-2-nitro-phenyl)-ethane-1,2-diamine, chlorhydrate
ethylenediamine, N-(5-methoxy-2-nitrophenyl)-,hydrochloride
1-methoxy-3-(2-aminoethylamino)-4-nitrobenzene, HCl

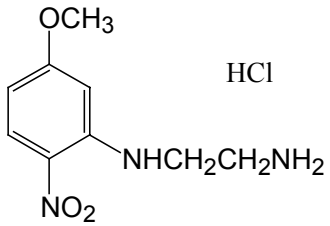
2.1.3. Trade names and abbreviations

Trade name : IMEXINE®FAD (Chimex)
COLIPA No. : B69

2.1.4. CAS/EINECS no.

CAS No. : 141973-33-3 (free base)
86419-69-4 (monohydrochloride)
EINECS : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_9H_{13}0_3\,N_3,\,HCl$
Mol weight : 247.68

2.1.7. Purity, composition and substance codes

All purity data relate to batch op.26

Purity

Titre as determined by potentiometry	:	99.2 - > 99.9% (w/w)
Water content	:	< 0.1% (w/w)
Ash content	:	< 0.1%(w/w)

Potential impurities

Reagents and reaction intermediates

2,4-dichloro-1-nitro-benzene	:	<100ppm (detection limit)
2,4-dimethoxy-1-nitro-benzene	:	<100ppm (detection limit)
N1,N3-bis-(2-amino-ethyl)-4-nitro-benzene-1,3-diamine	:	0.34%
4-methoxy-2-amino-1-nitro-benzene	:	260ppm

Three other impurities were detected. Mass spectra indicated that these could be isomeric forms of HC Yellow n° 9, but they could not be quantified because of the absence of authentic reference material.

Solvents :

Isopropanol	:	<100ppm
Methanol	:	<100ppm

Other:

Heavy metals	:	< 10ppm

2.1.8. Physical properties

Appearance	:	Yellow crystalline powder, almost odourless
Melting point	:	> 260°C
Boiling point	:	/
Flash point	:	273 °C
Density	:	0.23 g/cm^3
Rel. vap. dens.	:	/
Vapour Press.	:	/
Log P_{ow}	:	1.3 (method A8 84/449/EC); Calculated 1.9
Storage	:	Protect from light and moisture

2.1.9. Solubility

Water	:	0.1% in boiling water
Receptor Fluid*	:	65.8 µg/ml at 32°C

* receptor fluid used in percutaneous absorption study : Instamed® PBS buffer w/o Ca^{2+}, Mg^{2+} 9.55g/l

2.1.10. Stability

HC Yellow was stable in the hair dye formulations stored for two months at room temperature.

General comments on analytical and physico-chemical characterisation

* HC Yellow no° 9 is a secondary amine, and therefore, it is prone to nitrosation. No information is given on the nitrosamine content of the dye.

* The purity of the test material is determined by potentiometry. Chromatographic purity of the test material has not been reported.

* The purity of various batches of test material used for safety evaluation was 91.5% - 99%. The declaration of batch-to-batch similarity of the test substance given by the applicant is therefore not valid.

* The worst case of impurities in HC Yellow n° 9 (batch HB VI, 82) is assessed to be > 8%. The impurities in this batch have not been reported.

2.2. Function and uses

HC Yellow n° 9 will be incorporated into semi-permanent hair dyes at a maximum concentration of 0.5%.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	Journal Officiel de la Republique Francaise, norm no 03.021
Species/strain	:	Sprague Dawley Rat, OFA (SPF)
Group size	:	5 males + 5 females
Test substance	:	HC Yellow No. 9 suspended in 1.0% aqueous carboxymethylcellulose
Batch no	:	DG 1
Purity	:	/
Dose	:	700, 910, 1180, 1540 and 2000 mg/kg bw
Observ. Period	:	14 days
GLP	:	in compliance

Groups of 5 male and 5 female rats received a single dose of test substance at 700, 910, 1180, 1540 and 2000 mg/kg bw by gastric gavage. The animals were observed daily for 14 days after dosing. Body weights were recorded on days 1, 8 and 15 of the study. Macroscopic

examination of main organs was performed at autopsy. No histological examinations were performed.

Results
Mortalities occurred in all dose groups within 1 hour of treatment. Numbers of deaths were 2, 4, 5, 5, 5 males and 1, 1, 1, 3, 3 females at 700, 910, 1180, 1540 and 2000 mg/kg bw, respectively. At autopsy congestion and oedema of the lungs and oedema of the peritoneal cavity were reported in rats dying during the study period. No abnormalities were detected in animals sacrificed on day 14. The LD_{50} was reported to be 745mg/kg bw/day for males and 1609 mg/kg bw for females

Ref. : 1

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

Guideline	:	OECD 407 (1981)
Species/strain	:	Sprague Dawley rat, Crl:CD(SD) BR
Group size	:	6 males + 6 females
Test substance	:	HC Yellow N0. 9 suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	op.26
Purity	:	99.6 %
Dose	:	0, 30, 100 and 300 mg/kg bw/day
Exposure period	:	4 weeks (7 days per week)
GLP	:	In compliance

Groups of 6 male and 6 female rats were dosed with the test substance by gavage at 30, 100 and 300 mg/kg bw/day, 7 days a week for 4 weeks. The dosing solutions were prepared daily and protected from light until used. During the study, the animals were observed twice daily for clinical signs and mortality, and weekly for body weight and food consumption. Twenty-four hours after the final dosing, blood was sampled from the orbital sinus of fasted rats for haematology and blood biochemistry. Urine was collected overnight for urine-analysis from the fasting rats. At the end of the treatment period a full autopsy was conducted with macroscopic examination and recording of organ weights. Representative tissues were examined microscopically.

Results.
No deaths were reported in animals given 30 or 100 mg/kg bw/day. At the highest dose (300 mg/kg bw/day) on day 12, one female died, having exhibited piloerection, round back and emaciation for several days. The study authors concluded that although autopsy showed autolytic tissues, no relevant macroscopic or microscopic findings were noted. One female and 1 male died during week 4 at the highest dose (300 mg/kg bw/day). Neither showed any symptoms prior to death nor any macroscopic changes at necroscopy. Microscopically, both showed moderate

multifocal vacuolated hepatocytes. These were considered to be agonic changes by the study authors.
The main macroscopic findings related to the staining properties of the compound. Yellow to orange staining of the fur, tail, body extremities and urine was seen in all treated animals. Hypersalivation was reported in 4/6 females at 300 mg/kg bw/day.
The mean body weight gain and food consumption were comparable for all dose groups. Pre- and post-study ophthalmological observations did not differ.

There was a statistically significant decrease in blood glucose in males at the 100 and 300 mg/kg bw/day. In the females, there was a statistical significant increase in cholesterol and triglycerides at 300 mg/kg bw/day and inorganic phosphorus at 30 mg/kg bw/day. There was a statistically significant increase in urinary volume produced by the females at 300 mg/kg bw/day. There were no other statistically significant differences in the urine-analysis, haematological and biochemical parameters measured.
There was a statistically significant decrease in mean absolute and relative spleen weights in females treated with 300 mg/kg bw/day (-21% and –18% respectively). In rats, the spleen can maintain a low level of haematopoietic activity. This extramedullary haematopoiesis was evident histologically in the controls (3/6 male, 6/6 female) and the two lower dose groups (4/6 male, 6/6 female at each dose). At 300 mg/kg bw/day, 2/6 male and 1/6 female showed extramedullary haematopoiesis. This was not considered to be of toxicological importance by the study authors. They concluded it was possibly an artefact, due to variability in preparation of the tissue for histological examination. This seems to be an unsatisfactory explanation.
Minimal to moderate acidophilic globule incidence was noted in the cortical tubular epithelium in 3/6 males at 300 mg/kg bw/day and in one control male. Minimal to moderate basophilia (in some cases unilateral) were seen in 3/6 males at 300 mg/kg bw/day and in one control male and one female.
The other organ weights and microscopic findings were comparable with the controls. No treatment related microscopic findings were noted. The NOAEL was considered as 100 mg/kg day.

Ref. : 14

2.3.5. Repeated dose dermal toxicity

No data

2.3.6. Repeated dose inhalation toxicity

No data

2.3.7. Sub-chronic oral toxicity

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague Dawley rat, Crl:CD(SD) BR
Group size	:	10 males + 10 females
Test substance	:	HC Yellow No 9 suspended in 0.5% aqueous carboxymethylcellulose
Batch no	:	op.26
Purity	:	99.6 %
Dose	:	0, 25, 80 and 250 mg/kg bw/day
Exposure period	:	13 weeks (7 days per week)
GLP	:	In compliance

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 25, 80 and 250 mg/kg bw/day, 7 days a week for 13 weeks. The dosing solutions were analysed at the beginning and end of the study for stability and verification of homogeneity and concentration. During the study, the animals were observed daily for clinical signs and mortality. Body weight and food consumption were recorded weekly. During week 13, urine was collected overnight for urine-analysis. Blood was sampled from the orbital sinus of fasted rats for haematology and blood biochemistry. At the end of the treatment period a full autopsy was conducted recording organ weights, together with macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results
No deaths were reported in animals given 25 mg/kg bw/day. One female animal was found dead in week 1 (80mg/kg bw/day) and one in week 8 (250 mg/kg bw/day). In the absence of any relevant prior clinical signs the cause of death for both animals was considered to be either regurgitation of the material or a gavage error. The main macroscopic findings related to the staining properties of the compound. Yellow to orange staining of the fur, tail, body extremities and urine was seen in all treated animals. Hypersalivation was reported in 1/10 males treated at 25 mg/kg bw/day, in 4/10 males and females given 80 mg/kg bw/day and in all animals treated with 250 mg/kg bw/day. This was considered to be treatment related.
The mean body weight gain and food consumption were comparable for all dose groups. Pre- and post-ophthalmological observations did not differ.
There were some significant, but not dose-related, differences in the haematological and biochemical parameters measured. They were within the historical control range and therefore not considered to be of toxicological significance. The urine parameters were within the normal range.
Statistically significant increases in mean absolute and relative adrenal weights were found in females treated with 250 mg/kg bw/day (+14%). Higher mean absolute and relative kidney weights were noted in males given 250 mg/kg bw/day (+8% and +10% respectively). The relative weights only were statistically significant. Some other non-significant and non-dose related changes in organ weights were reported but considered to be of no toxicological significance. Other findings did not differ between dose groups and were not considered to be of toxicological importance. Compared with controls and the low dose group, there was a slight increase in the incidence and intensity of acidophilic globules in the tubular cortical epithelium and tubular basophilia of the male rat kidney at the higher doses, 80 and 250 mg/kg bw/ day. In this study, no degenerative or necrotic changes were reported. These changes were not reported in any female groups.
Tubular basophilia is reported to be a spontaneous lesion in this rat strain. The study authors suggested that the increased incidence seen was of minor toxicological significance.
Focal or multifocal coagulative hepatocellular necrosis was recorded in 1/10 males at 25 mg/kg bw/day, in 1/10 males and 1/10 females at 80mg/kg bw/day and in 2/10 males at 250mg/kg bw/day. This lesion was not seen in the control groups. The study authors suggested that this lesion was not treatment-related, as it can occur spontaneously in this strain.
Ulceration of the forestomach was reported in one female in the group treated with 80mg/kg bw/day. This lesion was not considered treatment related, since it can occur spontaneously in this strain.

The study authors concluded that the compound was clinically well tolerated at all dose levels. Microscopic findings of minor toxicological significance were noted in the kidneys of males

given 80 or 250 mg/kg bw/day, but these were considered to be specific sex-related phenomena to the male rat. The NOAEL was set at 250 mg/kg bw/day.
The SCCNFP considers that the NOAEL should be 80 mg/kg bw/day, based upon the changes in adrenal weights.

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 & corrosivity

2.4.1. Irritation (skin)

Guideline	:	Journal Officiel de la République Française, 21.02.82.
Species/strain	:	New Zealand Albino rabbits
Group size	:	3 (2 males and 1 female)
Test substance	:	HC Yellow No 9
Batch no	:	Op 10
Purity	:	/
Dose	:	0.5g moistened with 0.5ml of distilled water
GLP	:	In compliance

The substance (0.5g) was applied to a 6.25cm^2 area of intact and abraded skin of 3 rabbits. Occlusive patches were applied and left in place for a 24-hour period. Remaining test substance was removed by swabbing with cotton wool swabs. The skin was examined for erythema, eschar, formation and oedema at 1 and 48 hours after removal of the patches.

Results
Yellow coloration of the skin caused by the test material was seen at all treated sites, but interference with evaluation of erythema was not reported. Erythema was noted in two animals at both intact and abraded sites at the 1 hour observation. No oedema was reported.
The primary irritation index was calculated to be 0.3. The substance was non-irritant to the skin of the albino rabbit.

Ref. : 3

2.4.2. Irritation (mucous membranes)

Guideline	:	Journal Officiel de la République Française, 24.10.84.
Species/strain	:	New Zealand Albino rabbits
Group size	:	3

Test substance	:	HC Yellow No 9
Batch no	:	op10
Purity	:	/
Dose	:	0.1ml
GLP	:	In compliance

0.1ml of neat test substance was applied once to the lid of the right eye of each animal without rinsing. The left eye served as control. Ocular reactions were recorded at 1 hour and 1, 2, 3, 4 and 7 days after instillation.

Results
There was a dulling of the normal lustre of the cornea of one animal one hour after treatment. No other corneal effects were reported. Irritation of the conjunctivae was apparent in all animals 1 hour after treatment, returning to normal by day3. Inflammation and yellow staining of the iris was reported 1 hour after treatment and persisted in one treated eye for 24 hours. No further effects were reported during the study period only. According to the defined criteria of this study, the maximum irritancy score was 17.7/110. The substance was classified as slightly irritant to the rabbit eye.

Ref. : 2

2.5. Sensitisation

Guideline	:	Journal Officiel de la République Française, protocol N° T03-300
Species/strain	:	Dunkin-Hartley guinea pig
Group size	:	10 males + 10 females
Test substance	:	HC Yellow No 9
Batch no	:	DG 2
Purity	:	/
Concentration	:	induction : 0.1ml 50% Freund's complete adjuvant (FCA) 0.3g test substance x7 applications (Day 1-15) challenge : 0.3g test substance for 48 hours, occluded
GLP	:	In compliance

A preliminary intradermal study indicated that the test substance could be used neat without provoking an irritant response. Induction commenced with an intradermal injection (0.1ml) of FCA. The test substance (0.3g) was applied under a patch test for 48 hours, three times a week, every other day, for two weeks, and once at the start of week 3. After a 12 day rest period, the animals were challenged by a single topical application of the test substance (0.3g) under occlusive patch on the left flank for 48 hours. Appropriate controls were treated with vehicle alone on the right flank if necessary. The skin was examined for erythema and oedema, 1, 6 and 24 hours after removal of the challenge patches.

Results
One female animal died during the test period and was replaced; the cause of death was not established. Assessment of erythema was not possible due to skin staining. The compound was reported not to cause any cutaneous reactions.

Ref. : 4

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague Dawley Crl CD (SD) BR
Group size	:	25 females (mated)
Test substance	:	HC Yellow No 9 in 0.5% aqueous carboxymethyl cellulose
Batch no	:	op.26
Purity	:	99.6 %
Dose levels	:	0, 20, 70 and 250 mg/kg bw/day
Treatment period	:	Days 6-15 of pregnancy, inclusive
GLP	:	in compliance

Groups of 25 female rats were dosed with the test substance at 0, 20, 70 and 250 mg/kg bw/day by gavage on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality. Body weights and food consumption were recorded on days 0, 6, 9, 12, 15 and 20. The dams were sacrificed on day 20 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The foetuses were examined for bodyweight, sex and macroscopic external observations, and for skeletal and visceral abnormalities (half for each end point). The diet was analysed for homogeneity, stability and concentration.

Results
No deaths were reported. Clinical signs related to treatment were confined to yellow coloured urine in all animals given 70 and 250 mg/kg bw/day from day 7 to day 16 of pregnancy. One female rat, at the highest dose, displayed piloerection, round back and emaciation on day 20. No abortions occurred at any dose level. Compared with the controls, at 250 mg/kg bw/day, body weight gain (58g vs 70g). and food consumption (-12% to –15%), were lower over the treatment period. Weight gain and food consumption in the lower dose groups were comparable with the controls
At autopsy, no abnormalities were observed in any animal. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal body weights were similar for control and 20 and 250 mg/kg bw/day treatment groups. In the 70 mg/kg bw/day there was a slight increase in post implantation losses (7.3% vs 3.1%) but this was attributable to one female presenting 6/15 resorptions. This was not considered to be treatment related. The number of live foetuses was comparable with controls.
A small number of foetal malformations were observed, which were within the normal range. The treated groups did not differ significantly from control.
The test substance elicited maternal toxicity at 250 mg/kg bw/day but was not embryo-toxic or teratogenic at the doses tested. The NOEL for maternal toxicity was considered to be 70 mg/kg bw/day.

Ref. : 10

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1. Percutaneous absorption *in vitro*

Study 1

Guideline	:	/
Tissue	:	Human breast epidermis, heat-separated

Method	:	Franz diffusion cell (static)
Test substance	:	IMEXINE FAD, 0.45% in formulation
Batch no	:	Op 26
Purity	:	99.9 %
Dose levels	:	circa 40 mg formulation in the presence/absence of hair
Replicate cells	:	8/11
GLP	:	not in compliance

The skin penetration of HC Yellow n° 9 was evaluated in a static Franz diffusion cell using human epidermis prepared by heat-separation of previously frozen mammary skin. The test substance was prepared at a concentration of 0.45% in a formulation. Approximately 40 mg of the mixture was applied to $2cm^2$ of epidermal membrane for 30 minutes, with and without addition of finely chopped bleached hair and then excess washed off with distilled water and 2% sodium lauryl sulphate solution and dried. Four and 24 hours later the levels of substance were measured in the receptor fluid (Dulbecco's phosphate buffered saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study and with Chinese ink at the end of the study.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.08% of applied dose in the presence of hair and 0.4% of applied dose in the absence of hair.
This study did not include determination of recovery of the test substance. Physiological saline was used as the receptor fluid, which may not be adequate for a relatively lipophilic substance. The study is considered inadequate.

Ref. : 11

Study 2

Guideline	:	OECD draft 428 guideline n° 428
Test substance	:	HC YELLOW N° 9
Batch no	:	0503181
Purity	:	> 98.3 %
Tissue	:	Human abdominal dermatomed skin (kept at - 20°C)
Skin integrity	:	TEWL measurement
Method	:	Static diffusion cell 2 cm² / receptor compartment 3 ml
Receptor fluid	:	Instamed® PBS buffer w/o Ca^{++} Mg^{++} 9.55 g/l
Formulation tested	:	typical commercial formula
Dose formulation applied	:	20 mg/cm² (19.6 ± 0.2 mg/cm²)
Concentration of ingredient	:	0.41 % w/w (amount of HC Yellow n° 9 applied 79.8 ± 0.8 µg/cm²)
Replicate cells	:	4 skin donors, 2 cells/donor, 8 cells mounted, and interpreted
Duration of the contact	:	30 minutes
Duration of the diffusion	:	24 hours
Analytical method	:	HPLC with visible detection
Validation	:	limit of detection (0.005 µg/ml) and limit of quantitation (0.005 µg/ml) measured in the receptor fluid and in the extraction solvent of the tissue samples
Solubility in the receptor	:	verified at 32°C , 65.8 µg/ml

GLP : in compliance

The skin penetration of HC Yellow n° 9 was evaluated in a static Franz diffusion cell system. Human abdominal skin previously frozen was dermatomed to a constant thickness (570 ± 58 µm). The integrity of the skin was evaluated by the measurement of the TEWL (3.8 ± 1.0 g/m²/h), the skin surface temperature was monitored (31.2 ± 0.0 °C). The solubility of HC Yellow n° 9 in the receptor fluid (PBS buffer) was checked in the range of the concentration used. The test substance was prepared at a concentration of 0.41 % in a "commercial type" formulation. 20 mg/cm² of the formulation were applied to 2 cm² for 30 minutes. The excess from the skin surface was rinsed first with water, followed by a wash with 2 % aqueous sodium lauryl sulphate solution, again rinsed with water and finally dried with a cotton swabs.
24 hours after the application, the substance was measured in the receptor fluid using HPLC, in the horny layer collected by tape stripping (3 to 15 strips), in the epidermis and dermis altogether and in the remaining skin outside the application area. After assay of HC Yellow n° 9 in the washing material (skin excess) the mass balance of the study was calculated (88.6 ± 2.8 % of the applied dose)

Results
Most of the hair dye applied was recovered at the skin surface in the washing liquids (75.1 ± 8.86 %, 59.9 ± 6.96 µg/cm²). The quantity of test substance penetrating through the skin to the receptor fluid was 0.19 ± 0.21 % of the applied dose (0.15± 0.17 µg/cm²). The amount recovered in the horny layer was 7.56 ± 6.12 % (6.05 ± 4.90 µg/cm²). The epidermis and the dermis content was 5.70 ± 2.94 % of the applied dose (4.54 ± 2.32 µg/cm²). The amount found in the horny layer is higher than in the dermis and epidermis indicating a storage phenomenon of the hair dye in the *stratum corneum*.
The absorbed amounts of HC Yellow n° 9 (epidermis + dermis + receptor fluid) represents 5.89 ± 3.05 % of the applied dose (4.69 ± 2.41 µg/cm²) at the end of 24 hours of diffusion after a contact with the skin of 30 minutes.

Ref. : 13

2.8. Mutagenicity/Genotoxicity

2.8.1. Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471(1983 and revised draft 1994)
Species/strain	:	*S. typhimurium,* TA98, TA100, TA1535, TA1537, *E. coli* WP2 uvr A
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	IMEXINE FAD dissolved in DMSO
Batch no	:	op T 37
Purity	:	101 %
Concentrations	:	62.5-1000 µg/plate with and without metabolic activation.
GLP	:	in compliance

IMEXINE FAD has been investigated for gene mutation in *S. typhimurium* and *E. coli,* using the direct plate incorporation method and the preincubation method.

IMEXINE FAD has been investigated for gene mutation in *S. typhimurium* and *E. coli*. Liver S9 fraction from Aroclor 1254-treated rats was used as the exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline. The concentration range 62.5-1000 µg/plate was selected on the basis of maximum solubility.

Results
Without S9 mix : no dose related or biologically relevant increase in revertant numbers was observed, in any of the tester strains (*S. typhimurium and E. coli*).

With S9 mix : a significant but not reproducible increase in revertant numbers was observed only in the frameshift TA1537 tester strain. This increase exceeded the 2 fold criteria for positivity (x 2.1) in only one test. This sporadic mean increase is thought to be devoid of biological relevance because the frequency falls within the historical control values. It is not reproduced in the second assay and there is no trend for a dose-effect relationship.

Conclusions
The test is acceptable for evaluation.
Based on the reversion rate, and under the conditions of the assays performed, it is concluded that the test agent IMEXINE FAD is negative in the bacterial reverse mutation test.

Ref. : 6

***In vitro* Mammalian Chromosomal Aberration Test**

Guideline	:	OECD 473 (1983)
Species/strain	:	Chinese Hamster Ovary Cells
Replicates	:	Duplicate cultures, 2 independent tests
Test substance	:	IMEXINE FAD in DMSO
Batch no	:	Op 26
Purity	:	/
Concentrations	:	50-500µg/ml with and without metabolic activation.
GLP	:	in compliance

Preparation interval	Exposure period	Concentrations in µg/ml With and Without S9 mix		
24 h	24 h	50	250	500
48 h	48 h	50	250	500

Results

Toxicity
No relevant toxic effect was observed in the absence or in the presence of S9 mix. The top dose was chosen on the basis of solubility.

Structural chromosome aberrations

Experiment # 1
Without S9 mix : 24 h and 48 h harvest time

A statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control at 250 mg/ml in both independent assays. Moreover, while not statistically significant, there is a trend for a dose-response relationship.

With S9 mix : 24 h and 48 harvest time
No increase of the frequency of cells displaying structural chromosome aberrations was observed at any dose.

Experiment # 2
Without S9 mix : 24 h and 48 h harvest time
A statistically and biologically significant increase in the aberration rate was observed as compared to the corresponding solvent control at 500 mg/ml in both independent assays. Moreover, while not statistically significant, there is a trend for a dose-response relationship.

With S9 mix : 24 h and 48 harvest time
No increase of the frequency of cells displaying structural chromosome aberrations was observed at any dose.

Polyploidy
Not taken into account

The assay is acceptable for evaluation. IMEXINE FAD is considered positive for clastogenic activity in Chinese hamster ovary cell line in the absence of activation under the conditions of the tests.

Ref. : 7

2.8.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Mouse bone marrow micronucleus test - Schmid Method

Guideline	:	/
Species/strain	:	Mouse, Swiss Albino
Group size	:	10 males
Test substance	:	HC Yellow n° 9 in 20% DMSO
Batch no	:	HB VI, 82
Purity	:	91.5 %
Dose levels	:	0, 15, 25 and 35 mg/kg bw/day, twice, i.p.
Sacrifice Times	:	6 hours
GLP	:	Study not in compliance

HC Yellow n° 9 has been investigated for induction of micronuclei in the bone marrow cells of mice. The substance was administered by intraperitoneal injection twice at a 24 hour interval and groups of animals sacrificed at 6 hours after the last administration for harvest of bone marrow cells. No positive controls were used.

Results
Clinical signs of toxicity were reported (passivity, dyspnea, palpebral ptosis and piloerection).
There were no significant increases in micronucleated polychromatic erythrocytes in any of the test groups. The ratio of polychromatic to normochromatic erythrocytes was not reported.
No positive controls were used and therefore the sensitivity of the assay was not demonstrated.

The study is inadequate and unsuitable for evaluation

Ref. : 8

Mouse Bone Marrow Micronucleus Test - Salamone Method

Guideline	:	/
Species/strain	:	Mouse, Swiss Albino
Group size	:	10 males
Test substance	:	HC Yellow n° 9 in 20% DMSO
Batch no	:	HB VI, 82
Purity	:	91.5 %
Dose levels	:	0 and 40 mg/kg bw, i.p.
Sacrifice times	:	30, 48, 72 and 96 hours
GLP	:	not in compliance

A second micronucleus assay involved a single administration at 0 or 40 mg/kg bw by ip injection. The animals were sacrificed at 30, 48, 72 and 96 hours after the last administration for harvest of bone marrow cells. No positive controls were used.

Results
There were 21/40 deaths, occurring between 30 and 48 hours after administration of the compound. The main clinical signs reported were tremors, convulsions and catatonia.
There were no significant increases in the incidence of micronucleated polychromatic erythrocytes in the test groups at any sacrifice time. The ratio of polychromatic to normochromatic erythrocytes was not reported.
No positive controls were used and therefore the sensitivity of the assay was not demonstrated.
Individual animal data are not presented and it is not clear how many animals survived to each harvest point. The statistical power of the study is therefore questionable.
The study is inadequate and unsuitable for evaluation

Ref. : 8

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

Guideline	:	OECD draft guideline of 1991
Species/strain	:	Wistar rat, HanIbm:WIST (SPF)
Group size	:	4 males
Test substance	:	IMEXINE FAD suspended in PEG 400
Batch no	:	Op T 39
Purity	:	99.75 %
Dose levels	:	75 and 750mg/kg
Sacrifice times	:	2 hours: high dose group; 15 hours: all dose groups
GLP	:	in compliance

HC Yellow n° 9 has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. A preliminary toxicity study showed signs of toxicity but no deaths at 2000mg/kg bw and therefore this was used as the highest dose, in accordance with the OECD draft guideline. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 16 hours and for an additional high dose group after 2 hours. Hepatocytes were isolated and at least 3 cultures were established per animal. The hepatocytes were subsequently treated with ^{3}H-thymidine *in vitro.* Incorporation of radiolabel was assessed using autoradiography.

Results
Clinical Reactions
Adverse reactions to treatment were observed : apathy, reduction of spontaneous activity, eyelid closure, tremor. On the basis of the clinical signs observed, the Maximum Tolerated Dose (MTD)was estimated to be around 750 mg/kg

UDS assay
Negative control animals gave a group mean net nuclear grain (NNG) value of less than zero .Positive control animals gave a group mean positive NNG value.
Treatment with IMEXINE FAD at doses of 75 & 750 mg/kg yielded group mean NNG values less than zero and caused no significant increases, as compared to control, in the mean nuclear grain counts.

The study is adequate.
Data indicate that single oral gavage treatment of male rats dosed once with 75 & 750 mg/kg of IMEXINE FAD did not induce unscheduled DNA synthesis in hepatocytes isolated approximately 2 or 15 hours after dosing.
Under the experimental conditions, it is concluded IMEXINE FAD did not induce DNA repair activities detectable by this assay

Ref. : 9

Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474(21 July 1997)
Species/strain	:	Mouse, Swiss Ico (IOPS)
Group size	:	5 females and 5 males.
Test substance	:	IMEXINE FAD suspended in 0.5 % aqueous carboxymethylcellulose
Batch no	:	0503181
Purity	:	98.3 %
Dose levels	:	0 and 25, 50 or 100 mg/kg bw, fractionated oral gavage.
Sacrifice times	:	24 hours after last dosing
GLP	:	In compliance

IMEXINE FAD has been investigated for induction of micronuclei in the bone marrow cells of male or female mice. Dose levels were determined by a preliminary range finding study in which observable toxic effects and deaths were seen at 200 mg/kg bw/day in either sex. The substance was administered by gavage twice at a 24 hour interval and groups of animals sacrificed 24 hours after the last administration for harvest of bone marrow cells. Negative and positive controls were in accordance with the OECD guideline.

Results
Maximum Tolerated Dose (MTD):
The top dose of IMEXINE FAD was chosen on the basis of the clinical signs.
2 x 100 mg/kg.

Test doses:
IMEXINE FAD was administered by 1 single oral dose daily during 2 consecutive days. Dose: 100, 50 and 25 mg/kg .
1 sacrifice times was chosen. 24 h after the last dosing. Bone marrow smears were obtained from the positive control group 24 hours after single dosing only.
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.

Reactions to treatment:
No signs of clinical toxicity or mortality were observed.

Mean values of micronucleated PCE:
No statistically significant or biologically relevant increase in the incidence of micronucleated polychromatic cells over the concurrent vehicle control values were observed.

PCE/NCE ratio:
No statistically significant variation in the PCE/NCE ratio was observed in any dosage groups of male or female mice treated with IMEXINE FAD.

Under the conditions of the test it can be concluded that with IMEXINE FAD at doses at which no signs of clinical toxicity were recorded, and without significant variation in the PCE/NCE ratio, does not induce statistically significant increase in the frequency of PCE. The negative and positive controls gave the expected results.
Therefore, IMEXINE FAD is considered not to be clastogenic and/or aneugenic in this mouse bone marrow micronucleus test.

Ref. : 12

2.9. Carcinogenicity

No data

2.10. Special investigations

No data

2.11. Safety evaluation

NOT APPLICABLE

2.12. Conclusions

HC Yellow n° 9 is a secondary amine, and thus, it is prone to nitrosation. No information on the nitrosamine content of the dye and the dye formulation has been provided.
The purity of the test substance is determined by potentiometry. Chromatographic purity of the test material should be described. Different batches of test substances have been used for various tests. The purity of the compound in different batches appears to be 91.2 % - >99.9 %. The applicant's declaration of batch-to-batch similarity of the test substance is therefore not valid. Thus, the maximum impurity in the test material amounts to >8%. The worst case of impurities in HC Yellow n° 9 should be reported.

On the basis of the results of acute oral toxicity studies, the LD50 of HC Yellow n° 9 is 745 mg/kg/bw. The haematological results from the 28 day repeat dose and the 13 week subchronic oral toxicity studies were equivocal. There was an increase in adrenal weight in females in the subchronic oral toxicity study. The other histopathological changes were within the range of the historical controls. Maternal toxicity in the teratogenicity study was seen at 250 mg/kg bw/day. The NOAEL should be viewed as 80 mg/kg bw/day from the sub-chronic oral toxicity study.

HC Yellow n° 9 was slightly irritant when applied neat to the rabbit eye but not to rabbit skin. It has not been adequately tested for sensitising potential.

Percutaneous penetration has been investigated using human skin. Under the experimental conditions described, most of the hair dye was recovered in the washing liquids. Nevertheless the amount absorbed represents 4.69 ± 2.41 µg/cm² of the applied dose. The amount found in the horny layer is higher than in the dermis and epidermis, indicating a storage phenomenon at the surface of the skin.

HC Yellow n° 9 was tested in procaryotic cells for gene mutation in several tester strains *of S. typhimurium* and *E. coli* WP2 uvrA. The test agent is negative in the bacterial reverse mutation test. HC Yellow n° 9 is considered clastogenic in the absence of activation in the *in vitro* mammalian chromosomal aberration test.

The unscheduled DNA synthesis (UDS) test with Mammalian Liver Cells *in vivo* is acceptable and gives negative results.

Three *in vivo* genotoxicity studies - Mammalian Erythrocyte Micronucleus Test – have been supplied. However, the first two studies are not in accordance with the current OECD guidelines. They are inadequate and unsuitable for evaluation. The third *in vivo* Mammalian Erythrocyte Micronucleus Test supplied is acceptable for evaluation. Under the conditions of the test, it can be concluded that there was no evidence of induced chromosomal or other damage leading to the micronucleus formation in polychromatic erythrocytes of treated male or female mice . However, there is no evidence that the test agent has reached the target organ.

2.13. References

1. IFT, France. Report No. 306263 (June 1983)
2. Safepharm Labs Ltd,UK, Report No 109/270 (Mar 1994)
3. Safepharm Labs Ltd, UK, Report No 109/274 (Mar 1994)
4. IFT, France, Report No 311311 (Nov 1985)

5. CIT, France, Study No.10879 (Oct 1994)
6. CIT, France, Report No. 13149 MMJ (Sept 1995)
7. Toxicol Laboratories, UK, Study No. M/CCA/38502. (Mar 1994)
8. Department des "Controles Biologiques", France, Report No. CS/JC/92/116 (June 1992)
9. Cytotest Cell Research GmbH, Germany, Report No. 507500 (Aug 1995)
10. CIT, Report No 10878 RSR (May 1994)
11. L'Oreal, France. (June 1997)
12. CIT Report No. 20564 MAS (14 November 2000)
13. L'Oréal, Study No. 16114 (31 January 2001)
14. CIT, Study No. 9760 TSR (CIES 92125) (25 August 1993)

3. Opinion of the SCCNFP

The SCCNFP is of the opinion that the information submitted is inadequate to allow a risk assessment to be carried out.

Before any further consideration, the following information is required :

* chromatographic purity of HC Yellow n° 9;

* complete characterisation of the impurities in the worst case;

* nitrosamine content in various batches of HC Yellow n° 9 and in the hair dye formulations containing this chemical;

* data on genotoxicity/mutagenicity following the relevant SCCNFP opinions and in accordance with the Notes of Guidance.

4. Other considerations

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5. Minority opinions

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