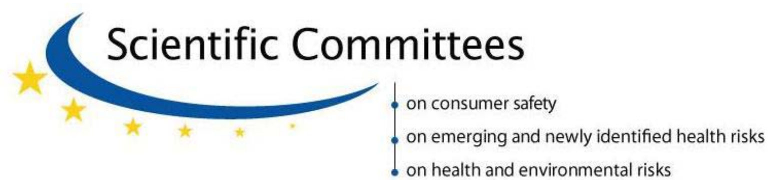
Scientific Committee on Consumer Safety

SCCS

# OPINION ON

# HC Yellow n° 9

## COLIPA n° B69




The SCCS adopted this opinion at its 7th plenary meeting

of 22 June 2010

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Gerhard Eisenbrand, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Kai Savolainen, Jacqueline Van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact

European Commission
Health & Consumers
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

© European Union, 2010

ISSN 1831-4767 ISBN 978-92-79-12739-7
doi:10.2772/23535 ND-AQ-09-011-EN-N

The opinions of the Scientific Committees present the views of the independent scientists who are members of the committees. They do not necessarily reflect the views of the European Commission. The opinions are published by the European Commission in their original language only.

http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

## ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Prof. G. Eisenbrand
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem (associated member)
Prof. M.P. Vinardell (rapporteur)
Dr. I.R. White

External experts

Dr. Mona-Lise Binderup COWI A/S, Denmark

Keywords: SCCS, scientific opinion, hair dye, B69, HC Yellow n° 9, directive 76/768/ECC, CAS 86419-69-4

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on HC Yellow n° 9, 22 June 2010

## TABLE OF CONTENTS

ACKNOWLEDGMENTS .......................................................................... 3

1. BACKGROUND .......................................................................... 5

2. TERMS OF REFERENCE .......................................................................... 5

3. OPINION .......................................................................... 6

4. CONCLUSION .......................................................................... 26

5. MINORITY OPINION .......................................................................... 27

6. REFERENCES .......................................................................... 27

## 1. BACKGROUND

Submission I for HC Yellow n° 9 with the chemical name 1-Methoxy-3-(ß-aminoethyl)amino-4-nitrobenzene hydrochloride has been submitted in February 1997 by COLIPA [1, 2].

Submission II for HC Yellow n° 9 has been submitted in January 2002 by COLIPA[2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its 24th plenary meeting on 24-25 June 2003 the opinion (SCCNFP/0680/03, final) with the conclusion that:

"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."*

Submission III for HC Yellow n° 9 was submitted by COLIPA in July 2005. According to this submission the substance is used in semi-permanent hair colouring products at a maximum concentration of 0.5%.

Submission III presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://ec.europa.eu/enterprise/sectors/cosmetics/files/doc/hairdyestrategyinternet_en.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

## 2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Safety (SCCS) consider HC Yellow n° 9 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 0.5% taken into account the scientific data provided?*

2. *Does the SCCS recommend any further restrictions with regard to the use of HC Yellow n° 9 in non-oxidative hair dye formulations?*

---

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

# 3. OPINION

## 3.1. Chemical and Physical Specifications

### 3.1.1. Chemical identity

#### 3.1.1.1. Primary name and/or INCI name

HC Yellow n° 9

#### 3.1.1.2. Chemical names

1-Methoxy-3-(β-aminoethyl)amino-4-nitrobenzene, hydrochloride
1,2-Ethanediamine, N-(5-methoxy-2-nitrophenyl)-, monohydrochloride (CAS name)
N-(5-methoxy-2-nitrophenyl)-1,2-ethanediamine, monohydrochloride
N-(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

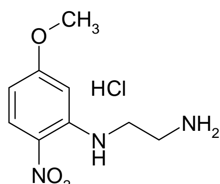
#### 3.1.1.3. Trade names and abbreviations

IMEXINE® FAD

#### 3.1.1.4. CAS / EC number

CAS: 86419-69-4 (monohydrochloride)
EC: 415-480-1

#### 3.1.1.5. Structural formula




#### 3.1.1.6. Empirical formula

Formula: $C_9H_{13}N_3O_3$, HCl

### 3.1.2. Physical form

Yellow crystalline powder, almost odourless

### 3.1.3. Molecular weight

Molecular weight: 247.68 g/mol

### 3.1.4. Purity, composition and substance codes

Purity

| Batch | Purity |
|---|---|
| Op. T37 | 101% |
| Op. T39 | 99.75% |
| Op. 26 | 99.6% |
| 0503181 | 98.3% |
| 0510071 | 98.5 |

UV-Vis spectra of all five investigated batches are in accordance with the proposed structure.
IR spectra of the investigated batches (0510071, 0503181, Op. 26) are in accordance with the proposed structure
$^{13}$C-NMR, $^{1}$H-NMR and mass spectra (batch 0510071, Op. 26) are in accordance with the proposed structure.

The purity has been determined by potentiometry for all batches except for batch 0510071 which have been analyzed by HPLC. The applicants claim that HPLC chromatograms are in agreement with the results of potentiometry.

Comment
The identity of batches OP T37 and OP T39 are not characterized according to state of the art.

Ref.: 00 (subm. III)

### 3.1.5. Impurities / accompanying contaminants

Impurities were determined by HPLC-DAD. Three of the possible impurities were identified as 2,4-dimethoxynitrobenzene, $N^1$,$N^3$-bis(2-aminoethyl)-4-nitrobenzene-1,3-diamine and 4-methoxy-2-amino-1-nitrobenzene by using adequate reference standards. Two further impurities were postulated to be isomers of $N^3$-(2-Aminoethyl)-$N^1$-[2-(5-methoxy-2-nitrophenylamino)-ethyl]4-nitrobenzene-1,3-diamine. They have been detected in batch 0510071 but have not been quantified accurately due to missing standards. The applicants calculated the amounts of these impurities to be < 0.1% each.
A further impurity of batch 0510071 was identified by LC-ESI-MS/MS to be 3-[2-Aminoethyl)-amino]4-nitrophenol and confirmed by an appropriate reference standard.

| Impurities | 0510071 | Op. 26 | Op. T37 | Op. T39 | 0503181 |
|---|---|---|---|---|---|
| 2,4-dichloronitrobenzene | <0.01 % | <0.01 % | <0.05 % | - | - |
| 2,4-dimethoxynitrobenzene | <0.01% | <0.01 % | <0.05 % | <0.01 % | 0.03 % |
| $N^1$,$N^3$-bis(2-aminoethyl)-4-nitrobenzene-1,3-diamine | 0.84 % | 0.34 % | - | - | - |
| 4-methoxy-2-amino-1-nitrobenzene | <0.01 % | 0.026 % | - | - | - |
| 3-[2-aminoethyl)-amino]4-nitrophenol | 0.21 % | - | - | - | - |
| $N^3$-(2-aminoethyl)-$N^1$-[2-(5-methoxy-2-nitrophenylamino)-ethyl]4-nitrobenzene-1,3-diamine (two isomers) | <?0.2 % | - | - | - | - |

- : no data available

Heavy metals (batch 0510071)

The heavy metal content was determined by ICP-OES and AAS

| | |
|---|---|
| Fe: | 22 ppm |
| Cu: | 37 ppm |
| Ni: | 3 ppm |
| Cr: | 7 ppm |
| Mo: | 8 ppm |

| | |
|---|---|
| Ti: | 1 ppm |
| Hg: | <0.1 ppm |
| Ag, Al, As, Ba, Bi, Cd, Co, Mn, Pb, Pd, Pt, Sb, Se, Sn, V, Zn: | < 1 ppm each |

Ref.: 00 (subm. III)

Comment
In its opinion of 2003, the SCCNFP requested specifically information on the nitrosamine content. However, the nitrosamine content has not been determined.

### 3.1.6. Solubility

Solubility was determined at 23°C after 24 h

Water: 5.21 g/L ± 0.11 at 20°C ± 0.5°C (EEC method A6)
Ethanol: <1 g/L
DMSO: <1 g/L

Comment
Solubility and partition coefficient have only been determined for Batch 0510071.

### 3.1.7. Partition coefficient (Log $P_{ow}$)

Log $P_{ow}$: 1.6 (Calculated) Batch 0510071
1.3 at 23°C (according to 84/449EEC method A8)

### 3.1.8. Additional physical and chemical specifications

| | |
|---|---|
| Melting point: | / |
| Boiling point: | / |
| Flash point: | / |
| Vapour pressure: | / |
| Density: | / |
| Viscosity: | / |
| pKa: | / |
| Refractive index: | / |
| UV_Vis spectrum (200-600 nm): | four maxima in absorbance at 206, 230, 252 and 322 nm. |

### 3.1.9. Homogeneity and Stability

Formulations of HC Yellow n° 9 in DMSO, 0.5% aqueous solution of methylcellulose (MC), 0.5% aqueous solution of carboxymethylcellulose_(CMC) and PEG were used for toxicological evaluation.

Homogeneity and stability tests were performed using batch 0510071.

To test homogeneity and stability, the content of HC Yellow n° 9 in the formulations was determined by HPLC-UV against reference standard solutions of HC Yellow n° 9. The analytical method was validated for all three vehicles.

<u>Homogeneity</u>

Homogeneity was examined in solutions of HC Yellow n° 9 in concentrations of 1 and 200 mg/mL (MC) and 10 mg/mL (CMC). The formulations were protected from light and under inert gas atmosphere. All samples were analyzed in duplicate on the day of preparation. The results showed the same variations as the analytical method itself, i.e. 1-5%.

Ref.: 13 (subm. III)

Stability
The formulations were stored for 4h (DMSO, methylcellulose) and 7d (carboxymethylcellulose) prior to use. PEG formulations are freshly prepared immediately before use.

Stability of solutions of HC Yellow n° 9 was examined in duplicate. The concentrations were 1 and 200 mg/mL (MC), 10 mg/mL (CMC) and 0.5 and 10 mg/mL (DMSO) directly after preparation and after 2 and 4 hours. The samples were stored at room temperature, protected from light and under inert gas atmosphere. The mean value of the homogeneity test was used as initial value for the stability test. The analytical results were within +2 and -7% of the initial value.

Ref.: 13 (subm. III)

Additional homogeneity and stability tests were performed for CMC solutions (batch Op. 26) at concentrations of 4 and 50 mg/ml and storage of up to nine days at +4°C and protected from light. Analysis was performed in duplicate by HPLC-UV. All formulations proved to be homogeneous within 6% of the nominal value.

Ref.: 10 (subm. III)

PEG formulations were not checked for stability because the formulations were freshly prepared immediately before use.

Ref.: 9 (subm. III)

Comment
Data on homogeneity of PEG formulations is missing.

### General Comments to physico-chemical characterisation

- With one exception the chemical identity of the batches is well characterized.
- Impurities account for up to 1.7%.
- Organic impurities have been determined using chromatographic techniques. However, not every impurity has been determined in each of the batches. In batch Op 26, which is used for testing repeated dose toxicity and teratogenicity, the organic impurities account for 0.37%.
- For the organic impurities, no toxicological data have been supplied. 4-Methoxy-2-amino-1-nitrobenzene is an aromatic amine which might have carcinogenic properties.
- HC Yellow n° 9 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. A possible nitrosamine content of HC Yellow n° 9 has not been determined.
  HC Yellow n° 9 should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
- Under the test conditions all formulations were stable and homogeneous.

### 3.2. Function and uses

HC Yellow n° 9 is an ingredient used in semi-permanent hair colouring products at a maximum concentration of 0.5%.

### 3.3. Toxicological Evaluation

#### 3.3.1. Acute toxicity

##### 3.3.1.1. Acute oral toxicity

***Taken from SCCNFP/0680/03***

| | |
|---|---|
| Guideline: | / |
| Species/strain: | Sprague Dawley Rat, OFA (SPF) |
| Group size: | 5 males + 5 females |
| Test substance: | HC Yellow n° 9 suspended in 1.0% aqueous carboxymethylcellulose |
| Batch: | 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 the 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 LD50 was reported to be 745 mg/kg bw/day for males and 1609 mg/kg bw for females.

Ref.: 1 (subm. II)

***New study***

| | |
|---|---|
| Guideline: | OECD 420 |
| Species/strain: | rat, Sprague-Dawley Rj: SD (IOPS Han) |
| Group size: | 1 female per group (sighting test)<br>5 females per group (main experiment) |
| Test substance: | HC Yellow n° 9 |
| Batch: | 0510071 |
| Purity: | 98.5% |
| Vehicle: | 0.5% suspension of methylcellulose |
| Dose: | 200, 500 mg/kg bw |
| Route: | oral gavage |
| GLP statement: | in compliance |
| Study period: | July 2004 – March 2005 |

In the sighting test, the test item was administered by oral route (gavage) at the dose level of 500 or 2000 mg/kg to one female per dose level. In the main experiment, the test item was administered by oral route (gavage) at the dose level of 200 or 500 mg/kg to two groups of five fasted female Sprague-Dawley rats (500 mg/kg group included the animal from the sighting test). The test item was prepared in a 0.5% suspension of methylcellulose and administered to the animals in a volume of 10 mL/kg. Clinical signs and mortality were

checked for a period of 14 days following the single administration of the test item. The animals were checked for body weight gain and were subjected to necropsy.

Results
Sighting test
At the dose level of 500 mg/kg, no mortality was observed, and the female showed hypoactivity, piloerection and dyspnea on day 1, hypoactivity and piloerection persisting on day 2.
At the dose-level of 2000 mg/kg, the female died on day 1; piloerection and tonic clonic convulsions were observed prior to the death. Accordingly, 200 and 500 mg/kg were chosen as doses for the main test.

Main experiment
At the 500 mg/kg dose-level, 3 of 4 females died on day 1. Piloerection, hypoactivity and/or tonic clonic convulsions were observed prior to death. In the surviving animal, piloerection and hypoactivity or sedation were noted on day 1 only.
At the 200 mg/kg dose-level, 1/5 females died on day 1. Piloerection, hypoactivity and dyspnea were observed prior to death. No clinical signs were noted in the surviving animals.
The overall body weight gain of the surviving animals was not affected by treatment with the test item. At necropsy, no apparent abnormalities were observed.

Conclusion
Under the experimental conditions, the oral LD50 of HC Yellow n° 9 was found to be between 200 and 500 mg/kg in rats. The maximum non-lethal dose-level is below 200 mg/kg.

Ref.: 1 (subm. III)

#### 3.3.1.2. Acute dermal toxicity

No data submitted

#### 3.3.1.3. Acute inhalation toxicity

No data submitted

### 3.3.2 Irritation and corrosivity

#### 3.3.2.1. Skin irritation

Guideline: /
Species/strain: New Zealand Albino rabbits
Group size: 3 (2 males and 1 female)
Test substance: HC Yellow n° 9
Batch: Op 10
Purity: /
Dose: 0.5g, moistened with 0.5 ml of distilled water
GLP: in compliance

The substance (0.5g) was applied to a 6.25 cm² 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 substance was non-irritant to the skin of the albino rabbit.

Ref.: 3 (subm. I)

**Submission III, 2005**

| | |
|---|---|
| Guideline: | OECD 404 (2002) |
| Species/strain: | New Zealand Albino rabbits |
| Group size: | 3 males |
| Test substance: | HC Yellow n° 9 |
| Batch: | 0510071 |
| Purity: | 98.5% |
| Dose: | 0.5 mL of the test item at the concentration of 5% (w/w) in a 0.5% suspension of methylcellulose in purified water |
| GLP: | in compliance |
| Study period: | May 2005 |

HC Yellow n° 9 was prepared at the concentration of 5% (w/w) in a 0.5% suspension of methylcellulose in purified water. Before preparation, the vehicle was degassed by sonication for at least 30 minutes. The test item was prepared as a suspension in the vehicle: the test item was ground to a fine powder, using a mortar and a pestle, and then mixed with the required quantity of vehicle.
The test item dosage forms were extemporaneously prepared under nitrogen atmosphere and were stored protected from light.
As possible irritant effects were anticipated, the dosage form was first evaluated on a single animal. Three different durations were investigated: 3 minutes, 1 hour and 4 hours.
Since the dosage form was not severely irritant on this first animal, it was then applied for 4 hours to two other animals.
Doses of 0.5 mL of the test item at the concentration of 5% were placed on a dry gauze pad, which was then applied to the anterior and posterior right flank (application for 1 and 4 hours) or the anterior left flank (application for 3 minutes) of the animals.
The gauze pad was held in contact with the skin by means of an adhesive semi-occlusive dressing and a restraining bandage.

Results
After a 3-minute exposure (one animal) a yellow coloration of the skin was noted during the study.
After a 1-hour exposure (one animal) a yellow coloration of the skin, which could have masked a well-defined or a discrete erythema, was noted from day 1 up to day 6.
After a 4-hour exposure (three animals) a yellow coloration of the skin, which could have masked a well-defined or a discrete erythema, was noted from day 1 up to day 2 or 3 (2/3 animals) and up to the end of the observation period (day 15; 1/3 animals).
Due to the coloration of the skin, the mean scores over 24, 48 and 72 hours for each animal were not calculable for erythema. No oedema was observed.

Conclusion
The yellow coloration of the skin could have masked a possible well-defined erythema. However, based on the coloration obtained and in the absence of other cutaneous reactions, the test substance at 5% is at most moderately irritant to rabbits.

Ref.: 2 (subm. III)

Comment

Because of skin staining, some irritant potential of 5% of HC Yellow n° 9 cannot be excluded.

### 3.3.2.2. Mucous membrane irritation

***Taken from SCCNFP/0680/03***

Guideline: /
Species/strain: New Zealand Albino rabbits
Group size: 3
Test substance: HC Yellow n° 9
Batch: 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 day 3. 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 (subm. I)

**Submission III, 2005**

Guideline: OECD 405 (2002)
Species/strain: New Zealand Albino rabbits
Group size: 3 males
Test substance: HC Yellow n° 9
Batch: 0510071
Purity: 98.5%
Dose: 0.5 mL of the test item at the concentration of 5% (w/w) in a 0.5% suspension of methylcellulose
GLP: in compliance
Study period: May 2005

The test item was prepared in the vehicle at the concentration of 5% (w/w). Before preparation, the vehicle was degassed by sonication for at least 30 minutes. The test item was prepared as a suspension in the vehicle: the test item was ground to a fine powder, using a mortar and a pestle, and then mixed with the required quantity of vehicle.
As possible irritant effects were anticipated, the dosage form was first administered to a single animal. Since the dosage form was not severely irritant on this first animal, it was then evaluated on two other animals.
A single dose of 0.1 mL of the test item at the concentration of 5% was instilled into the conjunctival sac of the left eye after gently pulling the lower lid away from the eyeball. The eyes were not rinsed after administration of the test item.
The eyes were examined approximately 1 hour, 24, 48 and 72 hours after administration of the test item. The study was ended on day 4 in the absence of persistent ocular reactions.

Results
A very slight chemosis (grade 1; 2/3 animals) and a very slight or slight redness of the conjunctiva (grade 1 or 2; all animals) were observed on days 1 and 2.
Mean scores calculated for each animal over 24, 48 and 72 hours were 0.0, 0.3 and 0.0 for chemosis, 0.3, 0.0 and 0.0 for redness of the conjunctiva, 0.0, 0.0 and 0.0 for iris lesions and 0.0, 0.0 and 0.0 for corneal opacity.

Conclusion
HC Yellow n° 9 at the concentration of 5% in 0.5% methylcellulose is slightly irritant when administered by ocular route to rabbits.

Ref.: 3 (subm. III)

### 3.3.3. Skin sensitisation

***Taken from SCCNFP/0680/03***

| | |
|---|---|
| Guideline: | / |
| Species/strain: | Dunkin-Hartley guinea pig |
| Group size: | 10 males + 10 females |
| Test substance: | HC Yellow n° 9 |
| Batch: | DG 2 |
| Purity: | / |
| Concentration: | induction: 0.1ml 50% Freund's complete adjuvant (FCA)<br>0.3g test substance x7 applications (Day 1-15)<br>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 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 (subm. I)

**Submission III, 2005**

**Local Lymph Node Assay (LLNA)**

| | |
|---|---|
| Guideline: | OECD 429 (2002) |
| Species/strain: | CBA/J mice |
| Group size: | 28 females (4 per group) |
| Test substance: | HC Yellow n° 9 |
| Batch: | 0510071 |
| Vehicle: | DMSO |
| Purity: | 98.5% |
| Concentration: | 0.05, 0.1, 0.25, 0.5 and 1% in DMSO |
| Positive control: | α-hexylcinnamaldehyde (HCA) at the concentration of 25% in DMSO |
| GLP: | in compliance |

Study period: April 2005

During the induction phase, the test item, vehicle or reference item was applied over the ears (25 µL per ear) for 3 consecutive days (days 1, 2 and 3). After 2 days of resting, the proliferation of lymphocytes in the lymph node draining the application site was measured by incorporation of tritiated methyl thymidine (day 6). 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.
α-hexylcinnamaldehyde (HCA) at the concentration of 25% in DMSO has been used as positive control

Results
The stimulation index was lower than 3 at all the five concentrations assayed. No dose-related effect was observed.
HC Yellow n° 9 at concentrations up to 1% did not induce delayed contact hypersensitivity in the murine Local Lymph Node Assay. No cutaneous reactions and no noteworthy increase in ear thickness were observed in the animals of the treated groups.

| Dose HC Yellow No 9 | SI |
|---|---|
| 0.05% | 1.15 |
| 0.1% | 1.20 |
| 0.25% | 1.12 |
| 0.5% | 1.73 |
| 1% | 1.12 |
| α-hexylcinnamaldehyde 25% | 16.02 |

Ref.: 4 (subm. III)

Comments
The concentrations tested were too low. A sensitisation potential cannot be excluded.

### 3.3.4. Dermal / percutaneous absorption

***Study 1, taken from SCCNFP/0680/03***

Guideline: /
Tissue: Human breast epidermis, heat-separated
Method: Franz diffusion cell (static)
Test substance: IMEXINE FAD, 0.45% in formulation
Batch: 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 2 cm² 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.

Ref.: 11 (subm. I)

Comment
The study did not follow a guideline and was not GLP compliant. It 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.

***Study 2, taken from SCCNFP/0680/03***

| | |
|---|---|
| Guideline: | OECD draft 428 |
| Test substance: | HC Yellow n° 9 |
| Batch: | 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 |
| Study period: | August 2001 |

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 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.: 12b (subm. III)

Comment
The test concentration was lower than the intended use concentration. The coefficient of variation (CV) of the absorption is high (51%). 9.51 μg/cm² (mean + 2 SD) should be used for MOS calculation.

### 3.3.5. Repeated dose toxicity

#### 3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

***Taken from SCCNFP/0680/03***

Guideline: OECD 407 (1981)
Species/strain: Sprague Dawley rat, Crl:CD(SD) BR
Group size: 6 males + 6 females
Test substance: HC Yellow n° 9 suspended in 0.5% aqueous carboxymethylcellulose
Batch: 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 statistically 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 bw/day.

Ref.: 20 (subm. III)

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

***Taken from SCCNFP/0680/03***

Guideline: OECD 408 (1981)
Species/strain: Sprague Dawley rat, Crl:CD(SD) BR
Group size: 10 males + 10 females
Test substance: HC Yellow n° 9 suspended in 0.5% aqueous carboxymethylcellulose
Batch: 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 (80 mg/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). Only the relative weights differed statistically significantly. 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 80 mg/kg bw/day and in 2/10 males at 250 mg/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 80 mg/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.

Ref.: 5 (subm. III)

Comment of the SCCS
In its opinion of 2003 (SCCNFP/0680/03), the SCCNFP considered that the NOAEL should be 80 mg/kg bw/day, based upon the changes in adrenal weights. In submission III, contemporaneous historical control data on adrenal weights at the testing laboratory were provided. These data demonstrate that the mean relative adrenal weight of the female historical controls is equal to the value found for females after 250 mg/kg bw/day HC Yellow n° 9. Consequently, the NOAEL is set to 250 mg/kg bw/day.

Ref.: 24 (subm. III)

#### 3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

### 3.3.6. Mutagenicity / Genotoxicity

#### 3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

**Bacterial Reverse Mutation Assay**

| | |
|---|---|
| Guideline: | OECD 471 (1983 and revised draft 1994) |
| Species/Strain: | *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA |
| Replicates: | triplicates in 2 independent experiments |
| Test substance: | Imexine FAD |
| Batch: | opT 37 |
| Purity: | 101% |
| Vehicle: | DMSO |
| Concentration: | 62.5, 125, 250, 500 and 1000 µg/plate without and with S9-mix |

| | | |
|---|---|---|
| Treatment: | experiment I: | direct plate incorporation with 48 – 72 h incubation without and with S9-mix |
| | experiment II: | direct plate incorporation with 48 – 72 h incubation without S9-mix |
| | and | preincubation method with 60 minutes preincubation 48 – 72 h incubation with S9-mix. |
| GLP: | in compliance | |
| Study period: | July - August 1995 | |

Imexine FAD was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli.* Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a preliminary toxicity test with *Salmonella* strains TA98 and TA100 and *Escherichia* strain WP2uvrA both without and with S9-mix. Toxicity was evaluated for 6 concentrations up to 1000 µg/plate on the basis of a reduction in the number of spontaneous revertant colonies and/or clearing of the bacterial background lawn. Since no relevant toxic effects were observed, 1000 µg/plate was chosen as the maximal concentration. Experiment I both without and with S9-mix and experiment II without S9-mix were performed with the direct plate incorporation method; experiment II with S9-mix with the preincubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Imexine FAD was poorly soluble in DMSO, the limit of solubility being approximately 10 mg/ml. Therefore, the highest possible dose was 1000 µg/plate. In the preliminary toxicity test no precipitate but a yellow colouration was observed when scoring at 50 to 1000 µg/plate. No toxicity was noted without and with S9-mix. A biologically relevant and dose dependent increase in the number of revertant colonies was not observed in any of the strains tested both without and with S9-mix. The small increase in the number of revertants found in TA1537 with S9-mix at 1000 µg/plate was considered not relevant since it was not reproducible and the value was within the range of the historical control data.

Conclusion
Under the experimental conditions used Imexine FAD was not mutagenic in this gene mutation tests in bacteria.

Ref.: 6 (subm. III)

### ***In vitro* chromosome aberration test**

| | |
|---|---|
| Guideline: | OECD 473 (draft) |
| Replicates: | duplicate cultures in 2 independent experiments |
| Cells: | CHO cells |
| Test substance: | Imexine FAD |
| Solvent: | DMSO |
| Batch: | Op 26 |
| Purity: | 99.6% |
| Concentrations: | 50, 250 and 500 µg/ml without and with S9-mix |
| Treatment: | treatment time not mentioned; harvest time 24 and 48 h after start of treatment both in the absence and presence of S9-mix |
| GLP: | in compliance |
| Study period: | March 1994 |

Imexine FAD has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Test concentrations were based on the results of a range finder experiment using suppression of mitotic index as the endpoint. Treatment times for both the range finder experiment and the main experiments were not

mentioned; harvest times were 24 and 48 h after the start of treatment. Two hours before harvest, each culture was treated with colcemid (final concentration 0.1 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the decrease in the mitotic index. Chromosome (metaphase) preparations were stained with 5% Gurrs Giemsa R66 and examined microscopically for chromosomal aberrations and the mitotic index. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Biologically relevant increases in the percentage cells with chromosomal aberrations were found at both harvest times in both experiments in the absence of S9-mix. At the highest concentrations, the increases were also statistically significant. In the presence of S9-mix no increases were observed.

Conclusion
Under the experimental conditions used Imexine FAD was genotoxic (clastogenic) in this *in vitro* chromosome aberration test with CHO cells.

Ref.: 7 (subm. III)

Comment
Treatment times for both the range finder experiment as for the main experiments were not mentioned.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

***In vivo* Mammalian Erythrocytes Micronucleus Test in mice**

Guideline: OECD 474 (1997)
Species/strain: mouse, Swiss Ico: OF1 (IOPS Caw)
Group size: 5 mice/sex/group
Test substance: Imexine FAD
Batch: 0503181
Purity: 98.3%
Vehicle: 0.5% aqueous carboxymethylcellulose
Dose level: 0, 25, 50, 100 mg/kg bw/day
Route: oral; 2 treatments separated by 24 h
Sacrifice times: 24 h after the second treatment.
GLP: in compliance
Study period: 27 July 2000 – 30 October 2000

Imexine FAD has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on a preliminary toxicity test measuring clinical signs and mortality recorded over a period of 48 h. In the main experiment mice were exposed orally to 2 treatments separated by 24 h of 0, 25, 50 and 100 mg/kg bw/day. Bone marrow cells were collected 24 h after the second dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/NCE). Additional animals were dosed for blood sampling to determine plasma levels of Imexine FAD. Blood samples were taken 4 h after the last treatment. Negative and positive controls were in accordance with the OECD draft guideline.

Results
In the preliminary toxicity test observable toxic effects (piloerection, mortality) were noted after the 2 applications of Imexine FAD at dose levels ranging from 2000 mg/kg/bw/day to 150 mg/kg bw/day. The top dose level was selected according to the criteria specified in the international guidelines: based on the toxicity level such that a higher dose level was expected to induce lethality.

No clinical signs or mortality were observed in the animals of both sexes in the main experiment. Treatment with Imexine FAD did not result in decreased PCE/NCE ratios compared to the untreated controls indicating that Imexine FAD did not have cytotoxic properties in the bone marrow. Biologically relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with Imexine FAD for both sexes, at any time point or dose level tested.

Conclusion
Under the experimental conditions used Imexine FAD did not induce micronuclei in bone marrow cells of treated mice and, consequently, Imexine FAD is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 8 (subm. III)

Comment
Compared to the untreated controls, treatment with Imexine FAD did not result in decreased PCE/NCE ratios (indicative for exposure of the target cells). However, in a parallel toxicokinetic study in mice, it was demonstrated that the animals were systemically exposed to HC Yellow n° 9 following oral administration (see ref 11). Consequently, the SCCS considers this *in vivo* micronucleus test acceptable.

***In vivo* unscheduled DNA synthesis (UDS) test**

| | |
|---|---|
| Guideline: | OECD 486 (draft from 1991) |
| Species/strain: | male rat; Wistar HanIbm: WIST (spf) |
| Group size: | 4 rats/group |
| Test substance: | Imexine FAD |
| Batch: | OP T 39 |
| Purity: | 99.75 % |
| Dose level: | 0, 75 and 750 mg/kg bw |
| Route: | oral gavage |
| Vehicle: | PEG 400 |
| Sacrifice times: | 2 h (high dose only) and 16 h post-treatment |
| GLP: | in compliance |
| Study period: | 22 March 1995 – 29 August 2000 |

Imexine FAD was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were based on results from a pre-experiment on acute toxicity evaluated at 1 and 24 h after start of treatment. In the main experiment mice were exposed orally to 0, 75 and 750 mg Imexine FAD /kg bw. Hepatocytes for UDS analysis were collected at 2 h (high dose only) and 16 h after administration of Imexine FAD. All animals from each group were perfused with collagenase for the collection of hepatocytes and establishment of cultures. After attachment of the cultures they were labelled for 4 h with 5 µCi/ml $^{3}$H-thymidine (specific activity 20 Ci/mmol). Evaluation of autoradiography was done 12-14 days after exposure. UDS was measured by counting nuclear grains and substracting the number of grains in one nuclear-sized area adjacent to the nucleus; this value is referred to as net grain count. Unscheduled synthesis was determined on 2 slides and 50 randomly selected hepatocytes per animal. Negative and positive controls were in accordance with the draft OECD guideline.

Results
In the a pre-experiment on acute toxicity with exposure up to 1000 mg Imexine FAD/kg bw, reduction of spontaneous activity, apathy and coloured urine a.o. were found. On the basis of these data the oral application of 750 mg/kg bw was estimated to be suitable. The viability of the hepatocytes was not substantially affected due to the *in vivo* treatment with Imexine FAD; variations were within the historical control data. No dose level of Imexine

FAD induced UDS in the hepatocytes of treated animals as compared to concurrent control values.

Conclusion
Under the experimental conditions used Imexine FAD did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 9 (subm. III)

## 3.3.7. Carcinogenicity

No data submitted

## 3.3.8. Reproductive toxicity

### 3.3.8.1. Two generation reproduction toxicity

No data submitted

### 3.3.8.2. Teratogenicity

***Taken from SCCNFP/0680/03***

Guideline: OECD 414 (1981)
Species/strain: Sprague Dawley Crl CD (SD) BR
Group size: 25 females (mated)
Test substance: HC Yellow n° 9 in 0.5% aqueous carboxymethylcellulose
Batch: op.26
Purity: 99.6 %
Dose levels: 0, 20, 70 and 250 mg/kg bw/day
Treatment period: Days 6-15 of pregnancy
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 diet was analysed for homogeneity, stability and concentration. 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).

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 (58 g vs 70 g) 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/d group, 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 was

observed which was 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 (subm. III)

Comment
The SCCS considers 70 mg/kg bw/d to be the NOAEL for maternal toxicity.

### 3.3.9. Toxicokinetics

The plasma toxicokinetic profile of HC Yellow n° 9 was investigated after single oral administration (gavage) to CD1 mice. Given the aim of the present study, the experimental conditions used (animal species, strain, age, dose-level, route, vehicle, dosage volume, dosage form preparation) were similar to those used in the mouse micronucleus test.
Twenty-four male and twenty-four female CD1 mice (about 6 weeks old) were placed into a single group and were treated once by oral administration (gavage) with the test item, HC Yellow n° 9 at the dose level 100 mg/kg bw. The test item was given as a suspension in 0.5% carboxymethylcellulose in a volume of 10 mL/kg. Blood samples were collected from the animals (three animals/sex/time point) as follows: 0, 0.25, 0.5, 1, 2, 3, 4, 8 and 24 hours, post-gavage. The plasma was analysed for test item levels by a HPLC/UV method.

Results
Following oral administration of the test item at a nominal dose level of 100 mg/kg in 0.5% carboxymethylcellulose (at 10 mL/kg) to CD1 mice, the mean (n = 3) (± standard deviation) plasma levels increased quickly to a maximum (Cmax) at 0.25 hour (tmax) post-gavage (2.36 ± 0.71 and 3.2 ± 1.62 µg/mL for males and females, respectively). Thereafter, levels decreased in a regular manner until the last quantifiable time-point at 4 hours for both sexes (0.385 ± 0.13 and 0.230 ± 0.22 µg/mL for males and females, respectively). The limit of quantification was 0.2 µg/mL. Exposure to the test item, as estimated by the Areas Under the Curve (AUC0-t and AUC0-∞), calculated using the log-linear trapezoidal rule (non-compartmental pharmacokinetics), were 3.02 and 4.08 µg/mL*h, respectively, for males and 3.79 and 4.18 µg/mL*h, respectively, for females (% of AUC extrapolated ≥ 20%). The terminal half-life (t1/2z), was 1.90 h for males and 1. 18 h for females.

Conclusion
The study showed that the animals were systemically exposed to HC Yellow n° 9 following oral administration at the dose level 100 mg/kg bw in 0.5% carboxymethylcellulose. The test item was above quantifiable limits (0.2 µg/mL) in the plasma until 4 hours post-dosing for both sexes, the plasma levels were characterized by a fast absorption phase and the Cmax was reached at 0.25 hour post-gavage.

Ref.: 11 (subm. III)

### 3.3.10. Photo-induced toxicity

#### 3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

#### 3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

### 3.3.11. Human data

No data submitted

### 3.3.12. Special investigations

No data submitted

### 3.3.13. Safety evaluation (including calculation of the MoS)

**CALCULATION OF THE MARGIN OF SAFETY**

**(HC Yellow n° 9)**

| | | | |
|---|---|---|---|
| **Absorption through the skin** | **A (mean + 2 SD)** | **=** | **9.51 µg/cm²** |
| **Skin Area surface** | **SAS ($cm^2$)** | **=** | **580 $cm^2$** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **5.52 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.09 mg/kg bw** |
| **No observed adverse effect level (maternal toxicity, teratogenicity, rat)** | **NOAEL** | **=** | **70 mg/kg bw/d** |
| **Margin of Safety** | **NOAEL / SED** | **=** | **761** |

### 3.3.14. Discussion

*Physico-chemical properties*
HC Yellow n° 9 is an ingredient used in semi-permanent hair colouring products.
Chemical identity of HC Yellow n° 9 is well documented.
Results of HPLC and Potentiometry are in agreement with respect to the purity of HC Yellow n° 9 batches.
The impurities of HC Yellow n° 9 account for 0.25 to 1.7%.
Organic substances which have been characterized with chromatographic techniques are the predominating impurities. No toxicity data have been supplied for these impurities.
Using a worst case calculation these impurities can reach a maximum concentration of 0.0085% in the product applied to the scalp.
HC Yellow n° 9 is a secondary amine and thus prone to nitrosation and formation of nitrosamines.
In its opinion of 2003, the SCCNFP requested specifically information on the nitrosamine content. However, the nitrosamine content has not been determined. HC Yellow n° 9 should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

*Skin/eye irritation and sensitisation*
In the skin irritation test, the test item caused yellow coloration of the skin which could have masked a possible well-defined erythema. Therefore, some irritant potential of 5% HC Yellow n° 9 cannot be excluded. In the absence of other cutaneous reactions, the test substance at 5% is at most moderately irritant to rabbit skin
HC Yellow n° 9 at the concentration of 5% in 0.5% methylcellulose is slightly irritant to the rabbit eye.
Due to the too low concentrations tested in the LLNA, a sensitisation potential cannot be excluded.

*Percutaneous absorption*

The absorbed amount 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 a 24 hours diffusion after contact with the skin for 30 minutes. Since the concentration tested was lower than the intended use concentration and the coefficient of variation (CV) of absorption is high, 9.51 µg/cm² (mean + 2 SD) is used for the MOS calculation.

*Toxicity*
In an acute oral toxicity study in rats the LD50 of the test item HC Yellow n° 9 was found to be between 200 and 500 mg/kg bw.
In the subchronic oral toxicity study in rats statistically significant increases in mean absolute and relative adrenal weights were found in females treated with 250 mg/kg bw/d. However, contemporaneous historical control data on adrenal weights at the test institute were provided. These data demonstrate that the mean relative adrenal weight of the female historical controls is equal to the value found for females after 250 mg/kg bw/d HC Yellow n° 9. Consequently, the NOAEL is set to 250 mg/kg bw/d.

In a teratogenicity study in rats the test substance elicited maternal toxicity at 250 mg/kg bw/d but was not embryotoxic or teratogenic at the doses tested. The NOAEL for maternal toxicity was considered to be 70 mg/kg bw/d. No reproductive toxicity study was provided.

*Toxicokinetics*
The toxicokinetics study showed that the animals were systemically exposed to HC Yellow n° 9 following oral administration at the dose level 100 mg/kg bw in 0.5% carboxymethylcellulose. The test item was above quantifiable limits (0.2 µg/mL) in the plasma until 4 hours post-dosing for both sexes, the plasma levels were characterized by a fast absorption phase and the Cmax was reached at 0.25 hour post-gavage.

*Mutagenicity/genotoxicity*
Overall, the genotoxicity of HC Yellow n° 9 is sufficiently investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Yellow n° 9 did induce gene mutations in a gene mutation tests in bacteria. A gene mutation test in mammalian cells was not performed. In an *in vitro* chromosome aberration study with CHO cells an increase in cells with chromosome aberrations was observed. The latter positive *in vitro* result for clastogenicity was not confirmed in an *in vivo* micronucleus test in mice. An *in vivo* unscheduled DNA synthesis test in rats was negative.
As the positive *in vitro* result was not confirmed in an *in vivo* test, HC Yellow n° 9 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

*Carcinogenicity*
No data submitted

## 4. CONCLUSION

Based on the information provided, the SCCS is of the opinion that the use of HC Yellow n° 9 as a non-oxidative hair dye ingredient at a maximum concentration on the head of 0.5% does not pose a risk to the health of the consumer.

A sensitising potential of HC Yellow n° 9 cannot be excluded.

HC Yellow n° 9 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

## 5. MINORITY OPINION

Not applicable

## 6. REFERENCES

**Submission I, 1997**

1. Monnot G. (1983) Essai de toxicité aiguë par voie orale chez le rat. Institut Français de Toxicologie (IFT), France. Report n° 306263, 30 June 1983
2. Guest R.L. (1994) Imexine FAD Op 10: primary eye irritation study in the rabbit. Safepharm Laboratories Ltd, UK. Project n° 109/270R, 10 March 1994
3. Guest R.L. (1994) Imexine FAD Op 10: primary skin irritation study in the rabbit. Safepharm Laboratories Ltd, UK. Project n° 109/274 R, 10 March 1994
4. Guillot J.P. et al. (1983) IFD 201-83, Imexine FAD - test pour l'évaluation du pouvoir sensibilisant par applications topiques chez le cobaye. Institut Français de Toxicologie (IFT), France. Report n° 311311, 10 November 1983
5. Richard J. et al (1994) 13-Week toxicity study by oral route in Rats - Imexine FAD. Centre International de Toxicologie (CIT), France Study n° 10879 TCR, 10 October 1994
6. Molinier B. (1995) Bacterial Reverse Mutation Test - Imexine FAD. Centre International de Toxicologie (CIT), France. Report n° 13149 MMJ, 26 September 1995
7. Kelly M.D. (1994) Imexine FAD - Metaphase Analysis of Chinese Hamster Ovary (CHO) Cells. Toxicol Laboratories Ltd, England. Study n° M/CCA/38502, March 1994.
8. Dossou K.G. (1993) Test du Micronoyau sur Moelle Osseuse de Souris Traitée in vivo par Voie Intraperitoneale. Produit testé B 69, l-Methoxy-3-(2-Aminoethyl-amino)-4-Nitrobenzene, HCl. Département des Contrôles Biologiques, L'OREAL, France. Report CS/JC/92/116, 12 June 1992
9. Volker W. (1995) In vivo/in vitro unscheduled DNA synthesis in rat hepatocytes with Imexine FAD. Cytotest Cell Research (CCR), Germany. Report n° 507500, 29 August 1995. First amendment to report, 18 September 1995
10. Richard J. (1994) Embryotoxicity/teratogenicity study by oral route in rats - Imexine FAD. Centre International de Toxicologie (CIT), France. Report 10878 RSR, 24 May 1994
11. Leclerc et al. (1997) Pénétration in vitro du colorant Imexine FAD Colípa B069 à travers l'épiderme humain isolé monté sur cellules de diffusion type Franz. Recherche Evaluation Sécurité Produits, Département de Chimie Analytique, L'OREAL, France. 23 June 1997

**Submission II, 2002**

12. Haddouk H. et al. (2000) IMEXINE FAD "Bone marrow micronucleus test by oral route in mice" C.I.T., Centre International de Toxicologie, France. Report 20564 MAS, 14 November 2000
13. Hueber F. et al. (2001) In vitro percutaneous absorption of HC Yellow n° 9 (Imexine FAD, Colipa B 069) ĽOréal Recherche, Direction des Sciences du Vivant, Evaluation Biologique et Sécurité, Biodisponibilité Cutanée et Métabolisme, France. Report 16114, 31/08/2001

14 Richard J. et al. (1993) IMEXINE FAD - 4-week preliminary toxicity study by oral route in rats. C.I.T., Centre International de Toxicologie, France. Report 9760 TSR, 25 August 1993

**Submission III, 2005**

References in italics are not submitted as full reports in the present dossier. They consist of reports for studies which were considered to be inadequate for submission [14-19], reports for preliminary toxicity studies [20, 25], or publications [21-23]; they can be provided upon request.

00. Jullien D. (2005) HC Yellow n° 9. Analytical file. L'Oreal, France
1. Sire G. et al. (2005). HC Yellow n° 9 (B069): Acute Oral Toxicity in Rats "Fixed Dose Method". CIT, France. Study n° 27944 TAR, 28 April 2005
2. Sire G. et al. (2005) HC Yellow n° 9 (B069) at the concentration of 5%: Acute Dermal Irritation in Rabbits. CIT, France. Study n° 29350 TAL, 3 May 2005
3. Sire G. et al (2005) HC Yellow n° 9 (B069) at the concentration of 5%: Acute Eye Irritation in Rabbits. CIT, France. Study n° 29351 TAL, 21 April 2005
4. Sire G. et al. (2005) HC Yellow n° 9 (B069): Evaluation of Skin Sensitization Potential in Mice using the Local Lymph Node Assay (LLNA). CIT, France. Study n° 27945 TSS, 20 April 2005
5. Richard J. et al. (1994) Imexine FAD: 13-Week Toxicity Study by Oral Route in Rats. CIT, France. Study n° 10879 TCR, 10 October 1994
6. Molinier B. et al. (1995) Imexine FAD: Bacterial Reverse Mutation Test. CIT, France. Study n° 13149 MMJ, 20 September 1995
7. Kelly M.D. (1994). Imexine FAD: Metaphase Analysis of Chinese Hamster Ovary (CHO) Cells. Toxicol Laboratories Ltd, England. Study n° M/CCA/38502, 17 March 1994
8. Haddouk H. et al. (2000). Imexine FAD: Bone Marrow Micronucleus Test by Oral Route in Mice. CIT, France. Study n° 20564 MAS, 14 November 2000
9. Völkner W. (1995) In Vivo / In Vitro Unscheduled DNA Synthesis in Rat Hepatocytes with Imexine FAD. CCR, Germany. Study n° 507500, 29 August 1995
10. Richard J. et al. (1994) Imexine FAD: Embryotoxicity/Teratogenicity Study by Oral Route in Rats. CIT, France. Study n° 10878 RSR, 24 May 1994
11. Klein E. et al (2005). HC Yellow N°9 (B069): Plasma Pharmacokinetics after Single Oral Administration to CD1 Mice. CIT, France. Study n° 27943 PAS, 25 March 2005
12a. Millequant J. (2005) Certificate of solubility of HC Yellow N°9 in hair colouring formulations devoid of other dye ingredients. L'Oréal, France. April 2005
12b. Hueber F. et al. (2001) In Vitro Percutaneous Absorption of HC Yellow N°9 (Imexine FAD, Colipa B069). L'Oréal, France. Study n° 16114, 31 August 2001
13. Mathieu D. et al. (2005) HC Yellow N°9 (B069): Validation of the analytical Method and Determination of Homogeneity and Stability of dosage Forms. CIT, France. Study n° 27942 AHS, 28 April 2005
14. Monnot G. Essai de toxicité aiguë par voie orale chez le rat. IFREB Study No. 306263, 1983
15. Guest R.L. Imexine FAD Op 10 : Primary Skin Irritation Study in the Rabbit. Safepharm Laboratories Ltd Study No. 109/274R, 1994
16. Guest R.L.. Imexine FAD Op 10: Primary Eye Irritation Study in the Rabbit. Safepharm Laboratories Ltd Study No. 109/270R, 1994
17. Guillot J-P. Test pour l'évaluation du pouvoir sensibilisant, par applications topiques, chez le cobaye. IFREB Study No. 311311, 1983
18. Dossou K.G. B 69 : Test du micronoyau sur moelle osseuse de souris traitée in vivo par voie intrapéritonéale. L'Oréal Study No. CS/JC/92/116, 1992
19. Leclerc. Pénétration in vitro du colorant IMEXINE FAD Colipa B069, à travers l'épiderme humain isolé monté sur cellules de diffusion type Franz. L'Oréal Trials No. 21/04/97, 05/05/97 and 13/05/97, 1997
20. Richard J. Imexine FAD: 4-Week Preliminary Toxicity Study by Oral Route in Rats. CIT Study No. 9760 TSR, 1993
21. Haschek W.M. Rousseaux C.G. The Kidney, in Fundamentals of Toxicologic Pathology, pp. 153-191. Academic Press, 2001
22. Schnellmann R.G. Toxic Responses of the Kidney. In: Casarrett and Doull's Toxicology – The basic Science of Poisons (Sixth Edition), pp 491-515. McGraw-Hill, 2001

23. SCCNFP/0680/03. Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers concerning HC Yellow N°9, COLIPA n° B69, 2003
24. Anonymous. Organ Weights: Historical Control Data for Rats used in 13-Week Toxicity Studies at CIT in 1994
25. Richard J. Imexine FAD: Preliminary Embryotoxicity Study by Oral Route in Rats. CIT Study No. 10877 RSR, 1994