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

CONCERNING

DIHYDROXYINDOLE

COLIPA n° : A111

adopted by the SCCNFP during the 23rd plenary meeting
of 18 March 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 Dihydroxyindole 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

Dihydroxyindole (INCI name)

21.2. Chemical names

Chemical name : 5,6-dihydroxyindole
CAS name : 1H-indole-5,6-diol
Synonyms : Indole-5,6-diol, Dopamine lutine

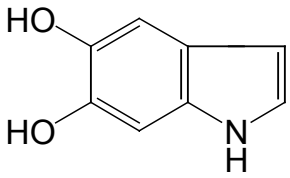
2.1.3. Trade names and abbreviations

Trade name : IMEXINE® OAY (Chimex)
COLIPA n° : A111
Substance code : P39

2.1.4. CAS no. / EINECS n°

CAS no : 3131-52-0
EINECS : /

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_8H_7NO_2$
Mol. weight : 149

2.1.7. Purity, composition and substance codes

Purity :

Titre as determined by potentiometry : 96-100%

Water content	:	≤ 0.4 %
Ash content (batch op 3X)	:	< 0.05% w/w
Heavy metals (batch op 3X)	:	< 20 ppm

Potential impurities :

Reagents and intermediate reaction products

4,5-dibenzyloxy-2-nitrophenylacetonitrile (batch op 3X)	:	< 0.02 %
4-hydroxy-3-oxo-9-aza-bicyclo-(4,2,0)-nonene-1 (batch op 1E)	:	< 0.2 %

Solvent residues

hexane, isopropyl ether, ethyl acetate (batch op 3X)	:	< 100 ppm
dichloroethane (batch op 3X)	:	0.25%
Chloride ions	:	< 0.4 %

2.1.8. Physical properties

Subst. Code	:	COLIPA A111
Appearance	:	A grey-white powder which rapidly darkens on exposure to air
Melting point	:	134 °C
Boiling point	:	no information
Density	:	1.28 g/ml
Rel. vap. dens.	:	no information
Vapour Press.	:	no information
Log P_{ow}	:	inadequate information

2.1.9. Solubility

Soluble in water at 12.3 % at 20 °C, in 96% ethanol at 10%, in chloroform at 0.1%

2.1.10. Stability

Dihydroxyindole is a very oxygen sensitive substance.

General comments on analytical and physico-chemical characterisation

* The solvent residues hexane and dichloroethane are CMR cat. 2 substances;
* Log Pow value provided (0.87), experimental protocol not specified;
* The purity of the test material has been determined by potentiometry. Thus, the purity information is inadequate.

2.2. Function and uses

Dihydroxyindole is used as a hair colorant without the need for an additional oxidant such as hydrogen peroxide. It forms coloured polymers in the presence of atmospheric oxygen. Dihydroxyindole will be incorporated in a hair dye formulation at a maximum concentration of 5% (typical concentration 0.5%). It is intended for weekly use with application of 25 ml formulation.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Guideline	:	OECD 401
Species/strain	:	Rat, Sprague-Dawley
Group size	:	5 male + 5 female
Test substance	:	Imexine OAY in 0.5% aqueous carboxymethyl cellulose
Batch no	:	Op 7 X (purity not stated in study report)
Dose	:	300, 378, 476 and 600 mg/kg bw in a volume of 10 ml/kg
Observ. Period	:	14 days
GLP	:	in compliance

Dose groups were selected on the basis of a preliminary range-finding study which indicated an oral LD50 between 400 and 500 mg/kg bw. Groups of 5 male and 5 female rats were fasted overnight before receiving a single dose of test substance by gastric gavage. The animals were observed 1 and 4 hours after dosing and then daily for 14 days. Bodyweights were recorded on days 0, 7 and 14, or at death. Macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results

No deaths occurred at 300 or 378 mg/kg bw. The two highest dose groups showed dose-related mortality (1M + 2F at 476 mg/kg bw; 3M + 4F at 600 mg/kg bw). Probit analysis indicated LD50 values (95% confidence limits) of 593 (451-701) mg/kg bw in male rats and 535 (420-682) mg/kg bw in female rats.

Deaths occurred within 5 days of dosing. Surviving animals appeared to show a dose-related decrease in weight gain, although statistical analysis was not presented in the study report. Clinical signs in surviving animals were hunched posture and piloerection in all animals, lethargy at 378 mg/kg bw and higher doses, and ataxia and ptosis at 600 mg/kg bw. No abnormal observations were reported at 5 days or more after dosing.

At autopsy, moribund animals exhibited red or haemorrhagic lungs and discoloration of the liver, kidneys or gastric epithelium, and gaseous distension of the stomach and small and large intestines. No abnormalities were observed at autopsy of rats dosed with 300 and 378 mg/kg bw. Rats dosed with 476 and 600 mg/kg bw had dark coloration of the kidneys.

Ref. : 1.1

Guideline	:	OECD 401
Species/strain	:	OF1 mouse, strain ICO:OF1 (IOPS Caw)
Group size	:	5 male + 5 female
Test substance	:	P39(1) in 0.5% aqueous carboxymethyl cellulose
Batch no	:	Batch 204/89 (purity not stated in study report)
Dose	:	200, 600 and 2000 mg/kg bw. in a volume of 10 ml/kg
Observ. Period	:	14 days
GLP	:	in compliance

Groups of 5 male and 5 female mice were fasted for 3-4 hours before receiving a single dose of test substance by gastric gavage. The animals were observed frequently for 4 hours after dosing and then daily for 14 days. Bodyweights were recorded on days 0, 5, 8 and 15, or at death. Macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results
No deaths occurred at 200 or 600 mg/kg bw. At 2000 mg/kg bw, the mortality was 90% (4M + 5F), all deaths occurring within 24 hours of dosing. The LD50 was reported as being between 600 and 2000 mg/kg bw.
Compared with the laboratory's historical control animal data, there was decreased bodyweight gain on day 4 in the animals dosed with 600 mg/kg bw but not at 200 mg/kg bw. Weight gain returned to normal by day 8. There were no clinical signs at 200 mg/kg bw. At 600 mg/kg bw, hypokinesia was observed during the first 24 hours after dosing; no abnormal observations were reported on subsequent days. The single surviving animal in the highest dose group appeared sedated on the day after dosing, but normal thereafter.
There were no apparent abnormalities at autopsy in animals found dead during the study, or sacrificed at termination.

Ref. : 1.2

2.3.2. Acute dermal toxicity

No data

2.3.3. Acute inhalation toxicity

No data

2.3.4. Repeated dose oral toxicity

No data

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
Species/strain	:	Sprague-Dawley rat, Crl:CD (SD) BR strain
Group size	:	16 male + 16 female
Test substance	:	P39 in aqueous solution
Batch no	:	Batch Op 3X (purity 99%)
Dose levels	:	0, 3, 10, 30 and 100 mg/kg bw/day, 7 days/week
Exposure period	:	92 - 96 days
Recovery period	:	4 weeks (6 males + 6 females from each dose group)
GLP	:	in compliance

Groups of 16 male and 16 female rats were dosed with the test substance by gavage at 0, 3, 10, 30 and 100 mg/kg bw/day, 7 days/week for 92 to 96 days. After 13 weeks treatment 6 rats of each sex from each dose group were kept for 4 weeks without treatment in order to observe for potential recovery. The animals were observed twice daily for clinical signs and mortality, and weekly for bodyweight and food and water consumption. At weeks 4 and 8, the animals were subjected to orbital bleeding for haematology and blood biochemistry analyses, and analyses were conducted on urine collected for 18 hour (overnight). These analyses were repeated at the end of the treatment and recovery periods, and full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the full treatment period.

Results

One animal (female, 3 mg/kg dose group) was found dead in week 13. Death was ascribed to an eye infection resulting from orbital bleeding. No treatment related mortalities occurred. Hypersalivation was noted in all males of the highest dose group during weeks 2 to 3, and in 15 of the 16 females of the highest dose group during weeks 3 to 5. Also in the 100 mg/kg bw/day group, black discoloration of the hair and tail and cage bedding was observed from week 10 until the end of the study. These observations were considered to be related to the presence of test substance or metabolites in the urine and/or faeces. Bodyweight gain and food and water consumption were similar for all dose groups.

Male rats treated with 30 and 100 mg/kg bw/day exhibited higher serum triglyceride at the weeks 4, 8 and 12 bleeds, which persisted after the recovery period. Other minor changes in haematological parameters and blood biochemistry were within the historical control range and not considered to be of toxicological significance. Overnight urine samples were dark coloured for males at 10 and 30 mg/kg bw/day and for all animals at 100 mg/kg bw/day, consistent with excretion of the dye and the reported observations on hair, tail and bedding. Colour was normal at the end of the recovery period. A dose-related increase in urinary ketones was evident in males at 10, 30 and 100 mg/kg bw/day, and to a lesser extent in females at the highest dose only. Reversibility was not complete. The authors concluded that, in the absence of other abnormalities, the ketonuria could be related to the mobilisation of free fatty acids, possibly related to the increase in plasma triglyceride.

No changes in organ weights were reported. Black discoloration of the kidneys was noted at autopsy, consistent with the microscopic observation of intracellular pigment accumulation in the cortical tubular epithelium. These changes occurred in some animals of both sex in the 30 and 100 mg/kg bw/day dose groups, and persisted at the end of the recovery period. No other histological abnormalities were reported.

On the basis of the increased triglyceride and accumulation of pigment in the kidney at 30 and 100 mg/kg bw/day, the study authors concluded that the dose level of 10 mg/kg bw/day was the NOAEL.
A significant change in urinalysis (ketones) was observed at 10 mg/kg bw/day, which could be related to the effects seen at higher doses, and therefore the NOEL is 3 mg/kg bw/day. Male rats appear to be more sensitive than females to the effects of this substance.

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)

Irritancy of 6% solution

Guideline	:	OECD 404
Species/strain	:	Hybrid New Zealand albino rabbit
Group size	:	6 male
Test substance	:	5,6-hydroxyindole: 6% in glycerol
Batch no	:	Batch op1E (purity not stated in study report)
Dose	:	0.5 ml
GLP	:	in compliance

Animal treatment was consistent with OECD guideline 404, except that the substance was applied to both intact and abraded skin for 24 hours.
0.5 ml of the test substance was applied to approximately 6.2 cm^2 intact and abraded (by means 3 incisions in the epidermis) skin of 6 male rabbits. Occlusive patches were applied and left in place for 24 hours. The skin was examined for erythema, eschar formation and oedema at 30 min and 48 hours after removal of the patches, i.e. 24 and 72 hours after commencement of treatment. An index of Cutaneous Primary Irritation was calculated from the mean scores at intact and abraded sites and each examination time.

Results
Slight erythema was observed at the intact application site for one rabbit after 24 hours. No other reactions were reported for intact skin. Mild to moderate reactions were observed in all rabbits at the abraded application sites after 24 hours, most of which had recovered by 72 hours.

The primary irritation score was 0.88 out of a maximum score of 8. According to the defined criteria, the test substance was reported to be slightly irritant to rabbit skin.

Ref. : 3.1

Irritancy of undiluted substance

Guideline	:	OECD 404 (1981)
Species/strain	:	New Zealand albino rabbit
Group size	:	1 male + 2 female
Test substance	:	IMEXINE OAY
Batch no	:	Batch op7X (purity not stated in study report)
Dose	:	0.5 g of pure test substance moistened with 0.5 ml distilled water
GLP	:	in compliance

0.5 g of moistened test substance was applied to 6.25 cm^2 of intact skin of 1 male and 2 female rabbits. Semi-occlusive patches were applied and left in place for 4 hours. Remaining test substance was removed by swabbing with cotton wool swabs soaked in diethyl ether. The skin was examined for erythema, eschar formation and oedema at 1, 24, 48 and 72 hours after removal of the patches. A Primary Irritation Index was calculated from the mean scores.

Results
Very slight erythema was observed on one of the two female rabbits after 1 hour and on the male rabbit after 24 and 48 hours. No other reactions were reported. Black staining due to the test substance was reported to be present throughout the study, but no comment was made on whether this interfered with the observations. The primary irritation index was 0.2 out of a maximum score of 8. According to the defined criteria, the test substance was reported to be a mild irritant to rabbit skin, not requiring a risk phrase under EEC labelling regulations.

Ref. : 3.2

2.4.2. Irritation (mucous membranes)

Guideline	:	OECD 405
Species/strain	:	Hybrid New Zealand albino rabbit
Group size	:	6 male
Test substance	:	5,6-hydroxyindole: 6% in glycerol
Batch no	:	Batch op1E (purity not stated in study report)
Dose	:	0.1 ml
GLP	:	in compliance

0.1 ml of the test substance was applied once to the right eye of 6 male rabbits, without rinsing. The left eye served as control. Ocular reactions were recorded at one hour and 1, 2, 3, 4, 7, days after instillation, and the Mean Ocular Irritation Score and Maximum Score of Ocular Irritation were calculated.

Results
Reactions were reported in the conjunctiva of all rabbits and the iris of 2 rabbits 1 hour after instillation. Observations on the iris returned to normal after 2 days, and on the conjunctiva by 7 days. No corneal reactions were reported. According to the defined criteria, the test substance was reported to be slightly irritant to the rabbit eye.

Ref. : 2

2.5. Sensitisation

Epicutaneous maximisation test

Guideline	:	AFNOR documentation FD nº TO3-300.
Species/strain	:	Dunkin-Hartley albino guinea pig
Group size	:	10 male + 10 female (no control group)
Test substance	:	5,6-hydroxyindole: 6% in glycerol
Batch no	:	Batch op1E (purity not stated in study report)
Concentrations	:	intradermal induction: 0.1 ml 50% Freund's complete adjuvant (FCA)
		topical induction : 7 application of 0.5 ml test substance over 15 d.
		challenge: 0.5 ml of test substance for 48 hours
GLP	:	in compliance

The study did not include a control group as specified by OECD guideline 406. Induction consisted of a single injection of FCA on day 1 and topical application of the test substance at 6% in glycerol on days 1, 3, 5, 8, 10, 12 and 15. Application sites were occluded for 48 to 72 hours until the next application. Following removal of the final patch (day 17), a period of 12 days was allowed before challenging with the same dose of test substance under occluded patch for 48 hours on a previously untreated area of skin. The skin was examined at 6, 24 and 48 hours after removal of the patches.

Results
Brown staining of the skin, due to the test substance, prevented proper evaluation of possible erythema.
The test is inadequate.

Ref. : 4.1

Magnusson and Kligman study

Guideline	:	OECD 406
Species/strain	:	Dunkin-Hartley albino guinea pig
Group size	:	20 treated + 10 control females
Test substance	:	IMEXINE OAY
Batch no	:	Batch op7X (purity not stated in study report)
Concentrations	:	intradermal induction: 0.1 ml 50% FCA
		0.1 ml 0.1% test substance in distilled water
		0.1 ml 0.1% test substance/FCA
		topical induction : 0.2-0.3 ml 50% test substance in distilled water
		challenge : 0.1-0.2 ml 10% test substance for 24 hours
GLP	:	in compliance

Induction commenced with three intradermal injections, of FCA, test substance, and a mixture of these two. One week later the induction process was completed with a single topical application of the test substance (50%) under occlusive patch to the shoulder region for 48 hours. An interval of 2 weeks was allowed after induction and then the animals were challenged by a single topical application of the test substance (10%) under occlusive patch on the right flank for 24 hours. Appropriate controls were treated with vehicle at all stages and the test substance-induced

animals received vehicle alone on the left flank. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results
A slight brown staining was observed due to the test substance. This was reported to preclude accurate assessment of erythema after the induction, but not after the challenge application. No adverse reaction was observed in any of the 20 treated guinea pigs and the test substance was not a sensitiser to guinea pig skin.

Ref. : 4.2

2.6. Teratogenicity

Guideline	:	OECD 414
Species/strain	:	Sprague-Dawley rat, Crl CD (SD) BR strain
Group size	:	25 females (mated)
Test substance	:	P39 in aqueous solution
Batch no	:	Batch Op 3X (purity 99%)
Dose levels	:	0, 20, 100 and 500/250 mg/kg bw/day
Treatment period	:	Days 6 to 15 of pregnancy, inclusive
GLP	:	in compliance

Groups of 25 female rats were dosed with the test substance by gavage on days 6 to 15 after mating. Dose levels were initially set at 0, 20, 100 and 500 mg/kg bw/day. Four of nine animals died 1 day after administration of the first dose at 500 mg/kg bw and the top dose was then reduced to 250 mg/kg bw for the remaining period. Sixteen animals allocated to the top dose received 250 mg/kg bw throughout the dosing period.
The dams were observed daily for clinical signs and mortality, and for bodyweight and food consumption on days 2, 6, 9, 12, 15 and 20. They 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 endpoint).

Results
No clinical signs were observed in control or 20 mg/kg bw/day dose groups. At 100 mg/kg bw/day, all females exhibited hypersalivation for the final 1 to 3 days of dosing. This was considered to be treatment related but of no toxicological significance. Clinical signs (piloerection, tremors, hypotonia, blackish coloured faeces, etc.) were reported in 4 of the 9 top dose group animals treated with 500 mg/kg bw for one day.
No deaths occurred in the 0, 20 or 100 mg/kg bw/day dose groups. All of the 9 animals treated with 500 mg/kg bw for one day died, including the 4 animals exhibiting clinical signs: 5 on day 7, 2 on day 9, 1 on day 10, a on day 12 and 1 on day 15. One top dose animal receiving 250 mg/kg bw on all dosing days died on day 9. All deaths were attributed to treatment. No abortions were observed.

Food consumption and bodyweight gain were reduced in the surviving top dose group animals, but not at 20 or 100 mg/kg bw/day. No macroscopic abnormalities were observed in the 20 or 100 mg/kg bw/day dams. In the highest dose group, 19 animals were reported to have blackish kidneys, and 8 with blackish stomach or intestines. Of the animals that died during the course of the study, 5 suffered autolysis and the others exhibited the macroscopic changes noted above.

The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal bodyweights were similar for control and treated groups. No treatment-related foetal anomalies or malformations were observed.

The authors concluded that the test substance was well tolerated by the pregnant female rat at dose levels of 20 and 100 mg/kg bw/day. The dose of 250 mg/kg bw/day was considered to be toxic for the pregnant female but not embryo-toxic or teratogenic. The NOAEL was defined as 100 mg/kg bw/day for materno-toxicity and 250 mg/kg bw/day for embryo-foetal development.

Ref. : 13

2.7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous Absorption *in vitro*

Guideline	:	/
Tissue	:	Human female abdominal skin, dermatomed
Method	:	Franz diffusion cell (static)
Test substance	:	P39, 4.6% in "GEL PROGRESS" formulation, batch number 48.791
Batch no	:	OP33 (radiochemical purity 93.4%)
Dose levels	:	1003.9 μg/cm^2 in the presence of 10.58 mg hair 805.8 μg/cm^2 in the absence of hair
Replicate cells	:	6
Analytical method:		HPLC with UV detection and radiolabelled detection
Stability	:	P39 is a very oxygen-sensitive compound. The study has been conducted in an anaerobic chamber (less than 1% oxygen) under N_2 flow.
GLP	:	not in compliance

Since Submission I, no new percutaneous study has been provided (only a clarification). The skin penetration of ^{14}C-radiolabelled COLIPA A111 was evaluated in a static Franz diffusion cell system using dermatomed human abdominal skin with and without addition of finely chopped uncoloured hair. The integrity of the skin was checked visually by a stereomicroscope instead of TEWL. The solubility of COLIPA A111 in the saline receptor fluid was not evaluated although it was assumed taken into account its solubility in water (12.3 %) and its rather hydrophilic character.
The test substance was prepared at a concentration of 4.6% in a formulation. In absence of hair, 17.51 ± 3.31 mg/cm^2 of formulation were applied on the skin for 30 min, corresponding to 805.08 ± 152.09 μg/cm^2 of the test compound. In presence of hair, those figures were 21.83 ± 4.34 mg/cm^2 and 1003.90 ± 199.79 μg/cm^2, respectively. Then, the skin surface excess was washed off with 2% sodium lauryl sulphate solution, rinsed with water and finally dried. Four hours later, the levels of radioactivity were measured in the epidermis (without separating SC), the dermal layer, the receptor fluid and, where applicable, in the hair. After assay of COLIPA A111 in the washing material (skin excess) the mass balance of the study was calculated.

Results
According to Submission I, after 4 hours 30 min., the mean quantity of test substance penetrating through the epidermis to the dermal layer and receptor fluid corresponded to 2.24 μg/cm^2 in the presence of hair and 2.52 μg/cm^2 in the absence of hair. However, the SC and viable epidermis have not been separated. As no new percutaneous absorption study has been provided in Submission II, the amount of the test substance detected in epidermis (23.41 μg/cm^2 in the

presence of hair and 19.00 μg/cm^2 in the absence of hair) must be considered in order to know the global amount penetrated through the skin. The recovery of the test substance is 90.67 ± 6.96% in the presence of hair and 87.37 ± 3.88% in the absence of hair. The duration time of the study is considered insufficient to allow a complete penetration to the receptor fluid.
The absorbed amounts of COLIPA A111 (epidermis + dermis + receptor fluid) represent 25.65 μg/cm^2 in the presence of hair and 21.52 μg/cm^2 in the absence of hair, at the end of 4 hours of diffusion after a contact with the skin of 30 minutes.

Ref. : 14

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline	:	OECD 471
Species/strain	:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, E. Coli WP2 uvrA-
Batch no	:	OAY OP7X
Purity	:	purity not stated
GLP	:	in compliance

Liver S9 fraction from Sprague Dawley liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results

In the first experiment	:	- S9	:	negative in all tester strains
		+ S9	:	negative in all tester strains
Second experiment	:	- S9	:	negative in all tester strains
		+ S9	:	negative in all tester strains

Conclusions
Based on the reversion rate, it is concluded that the test substance A 111 does not show evidence of mutagenic activity in two bacterial test systems in the presence or in the absence of activation.

Ref. : 6

***In Vitro* Mammalian Chromosome Aberration Test**

Guideline	:	OECD 473
Species/strain	:	Chinese Hamster Ovary (CHO) cells
Replicates	:	yes
Test substance	:	P34OP3X
Purity	:	99 %, impurities 0.2 %
Exposure time	:	- S9 : 21 h + S9 : 3 h
GLP	:	in compliance

Liver S9 fraction from liver rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system.

Results
COLIPA A111 has been investigated for induction of chromosomal aberrations in Chinese Hamster Ovary cells. Liver S9 fraction from rats (origin not described) was used as the exogenous metabolic activation system.
Without S9 : clastogenic activity was observed for the highest dose in both experiments.
With S9 : clastogenic activity was observed for 2 doses in the second experiment.

Conclusions
This study is adequate, the test agent displays clastogenic properties both in the presence or absence of activation systems.

Ref. : 7

2.8.2. Mutagenicity/Genotoxicity *in vivo*

In Vivo Mammalian Erythrocyte Micronucleus Test

Guideline	:	OECD 474
Species/strain	:	Mouse, Albino BKW mice
Group size	:	5 male + 5 female
Test substance	:	Imexine OAY OP 7X, batch OP 7 X
Batch no	:	purity not given
Dose levels	:	400 mg/kg bw, single intragastric gavage
Sacrifice times	:	24, 48 and 72 hours after dosing
GLP	:	in compliance

COLIPA A111 has been investigated for induction of micronuclei in the bone marrow cells of Albino BKW mice. The substance was administered once by single intragastric gavage at 400 mg/kg bw and the bone marrow harvested after 24 ,48 and 72 hours. Negative and positive controls were in accordance with the OECD guideline.

Results
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
Groups of mice treated with COLIPA A111 did not exhibited a significant variation of the PCE/NCE ratio.

Conclusions
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 treated mice.
However, it should be noticed that the absence of variation in the PCE/NCE ratio does not allow to determine if the test agent has reached the bone marrow. In addition, individual values for PCE and NCE fall in a very large range which may have influenced the results observed so far. The study is considered inadequate.

Ref. : 8

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline	:	OECD 486
Species/strain	:	hepatocytes of male Wistar rats
Test substance	:	KN 17
Batch	:	OP3X , purity = 99 % (potentiometer)
Exposure time	:	2-4 h and 12-14 h
GLP	:	in compliance

COLIPA A111 has been investigated for induction of unscheduled DNA synthesis in Wistar rats hepatocytes at 2 doses of 316.2, and 1000 mg/kg.
Positive controls are in accordance with OECD guideline and UDS analysed by autoradiography.

5 animals were used per dose/time sampling.

Results
No evidence of UDS induced by the test agent was observed.

Conclusions
This study is adequate and the results negative, the test agent does not induce UDS under *in vivo* conditions.

Ref. : 9

General Conclusions

* A111 tested in procaryotic cells for gene mutation in 5 tester strains . The results of the bacterial gene study demonstrated the absence of mutagenic properties at the gene level in both bacterial species.
* The *in vitro* test for clastogenicity in CHO cells is considered positive with and without metabolic activation.
* The UDS *in vivo* on rats hepatocytes is negative.
* The *in vivo* micronucleus test in Albino BKW mice gave negative results. However, it should be noticed that the absence of variation in the PCE/NCE ratio does not allow to determine if the test agent has reached the bone marrow. In addition, individual values for PCE and NCE fall in a very large range which may have influenced the results observed so far.
* The substance induces structural chromosome aberrations in mammalian cells *in vitro* but not gene mutation in bacteria. One *in vivo* study (*in vivo/in vitro* rats hepatocytes) indicates that the compound is not an *in vivo* somatic cell genotoxic agent.

2.9. Carcinogenicity

No data

2.10. Special investigations

Dihydroxyindole is a naturally occurring substance and is a precursor of melanin. It has therefore been the subject of some investigations into potential therapy for melanoma and other pigment disorders. Two of these studies have been included in the COLIPA submission. They are in the form of papers from the peer-reviewed scientific literature and are not conducted to recognised guidelines or to GLP.

A study conducted in mouse L fibroblasts and Cloudman S-91 melanoma cells, used incorporation of ^{3}H-thymidine into protein as the endpoint for cytotoxicity. The concentration of COLIPA A111 that inhibited protein synthesis in melanoma cells to 50% of control was in the region of 10^{-5} to 10^{-4}M. Dopa was approximately 10-fold less potent. It was reported that COLIPA A111 was also cytotoxic to fibroblasts, but the concentration range was not specified. In contrast, dopa was not found to be cytotoxic to fibroblasts.

Ref. : 10

A second study was conducted using a number of human melanoma cell lines and human fibroblast strains derived from skin biopsies, with a clonal assay as the endpoint for cytotoxicity. COLIPA A111 reduced survival at similar concentrations for melanoma and fibroblastic cell lines (30 – 77 μM reduced survival to 50% of control). Other dihydroxy compounds (catechol, epinephrine and α-methyl dopa) exhibited similar potency to COLIPA A111 in cytotoxicity towards fibroblasts, but were selectively more cytotoxic to melanoma cells.

Ref. : 11

Remark
These studies are not informative for the safety evaluation of COLIPA A111 in hair dye formulations.

2.11. Safety evaluation

CALCULATION OF THE MARGIN OF SAFETY

(Dihydroxyindole)
(air oxidative)

Based on a usage volume of 25 ml, containing at maximum 5 %

Maximum absorption through the skin	**A (μg/cm²)**	**=**	**25.65 μg/cm²**
Typical body weight of human		**=**	**60 kg**
Skin Area Surface (scalp)	**SAS**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**17.96 mg**
Systemic exposure dose (SED)	**SAS x A x 0.001/ 60**	**=**	**0.299 mg/kg**
No observed effect level (mg/kg) (rat, 13 weeks gavage)	**NOEL**	**=**	**3 mg/kg**

Margin of Safety	**NOEL / SED**	**=**	**10**

2.12. Conclusions

The overall package of tests is adequate and most have been conducted to GLP and appropriate guidelines.

The substance was moderately toxic by ingestion. A 13-week oral repeat dose study in rats showed intracellular pigment accumulation in the kidneys, elevated serum triglyceride levels and increased urinary ketones, particularly in male rats. These effects appeared to be treatment-related and did not resolve by the end of a 4-week recovery period. The NOAEL for the pigment accumulation and elevated triglyceride levels was 10 mg/kg bw/day and this was reported by the authors as the NOAEL for the study. However, increased urinary ketones were seen at 10 mg/kg bw/day and it is possible that they provided a sensitive marker of effect. On this basis, a NOEL of 3 mg/kg bw/day is used for the calculation of the Margin of Safety.

The NOAEL for maternal toxicity in a teratogenicity study with dosing over the period of organogenesis was an order of magnitude higher than that produced in the 13-week study and there was no evidence of embryotoxicity, teratogenicity or developmental effects under the conditions of the study.

A111 was slightly irritating to the rabbit eye and skin at a concentration of 6%, which is similar to the maximum concentration of 5% for human use.
It has not shown evidence of sensitisation reactions.

Percutaneous penetration has been investigated using human skin *in vitro*. Although broadly in line with recommendations for such studies the duration of the experiments did not allow sufficient time for penetration. As no new percutaneous absorption study has been provided, it is considered a value of percutaneous absorption of 25.65 $\mu g/cm^2$.

The substance induced chromosomal aberrations in mammalian cells *in vitro* but not gene mutations in bacteria. One *in vivo* study (*in vivo/in vitro* on rat hepatocytes) indicates that the compound is not an *in vivo* somatic cell genotoxic agent.

Thus overall, there are concerns about the value of the NOEL in the 13-week study as well as on the results obtained from mutagenicity/genotoxicity studies and on the duration of the skin penetration studies. The margin of safety does not appear to be adequate.

2.13. References

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12. Miranda, M., Bonfigli, A., Zarivi, O., Manilla, A., Cimini, A.M. and Arcadi, A., Mutagenesis 2: 45-50 (1987).
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3. Opinion of the SCCNFP

Dihydroxyindole is clastogenic *in vitro* with and without metabolic activation but is not a bacterial mutagen. One *in vivo* study (*in vivo/in vitro* rat hepatocytes) indicates that the compound is not an *in vivo* somatic cell genotoxic agent.

The NOEL from a rat 13-week study was 3 mg/kg/day. *In vitro* studies indicate a maximum value of 25.65 $\mu g/cm^2$ for percutaneous penetration considering the epidermis, dermis and receptor fluid compartments. Comparing the NOEL with the maximum systemic exposure dose in humans indicated a margin of safety of 10, which is not adequate.

The SCCNFP is of the opinion that Dihydroxyindole is not suitable for use in hair dyes.

4. Other considerations

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5. Minority opinions

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