EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

Isatin

COLIPA N° A129

Adopted by the SCCP during the 3rd plenary meeting
of 15 March 2005

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1. BACKGROUND

Submission II is an updated dossier in line with the second step of the strategy on the evaluation of individual hair dyes http://pharmacos.eudra.org/F3/cosmetic/doc/HairDyeStrategyInternet.pdf

2. TERMS OF REFERENCE

1. *On the basis of currently available information, the SCCP is asked to assess the risk to consumers of Isatin (2,3-Indolinedione, COLIPA[1]-- no A 129) when used in hair dye formulations.*
2. *Does the SCCP recommend any further restrictions with regard to the use of Isatin (2,3-Indolinedione, COLIPA no A 129) in hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

Isatin (INCI name)

3.1.1.2.	Chemical names

2,3-Indolinedione
1-H-Indole-2,3-dione
Indolinedione-2,3
Indole-2,3-dione
2,3-diketoindoline
2,3-dioxo-2,3-dihydroindole
2,3-dioxoindoline
Isatic acid lactam
Isatin(e)
Isatinic acid anhydride
o-Aminobenzoylformic anhydride

3.1.1.3.	Trade names and abbreviations

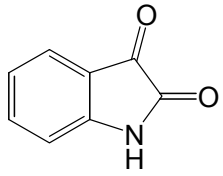
Trade name : Isatin
COLIPA n° : A129

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3.1.1.4.	CAS / EINECS number

CAS : 91-56-5
EINECS : 202-077-8

3.1.1.5.	Structural formula



3.1.1.6.	Empirical formula

Formula : $C_8H_5NO_2$

3.1.2.	Physical form

Orange crystalline powder

3.1.3.	Molecular weight

Molecular weight : 147.14

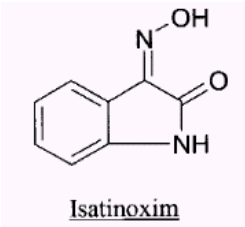
3.1.4.	Purity, composition and substance codes

Identification, purity and impurities

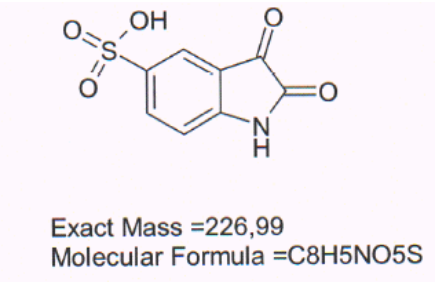
Batch No.	**5059972**	**503-3427**	**030031P050**
IR spectrum	In accordance with the proposed structure		
UV spectrum	UV spectra are similar		
Mass spectrum	Compatible	-	Compatible
^{1}H and ^{13}C NMR spectrum	In accordance with the proposed structure		
Purity[a] (UV-Vis spectro-photometric determination)	99.8 g/100 g	99.7 g/100 g	100.4 g/100 g
Water content (K.F. method)	-	-	0.2 g/100 g
Loss on drying	-	-	0.3 g/100 g
Impurities			
Isatinoxim	0.22 g/100 g	-	0.37 g/100 g
Impurity A[b]	n.d.	-	0.25 g/100 g
Residual solvents	n.d.	n.d.	Acetone <100 ppm

[a] : determined using indolinedione standard from Fluka.
[b] : relative concentration based on HPLC-peak area
n.d. : not detected (< 100 ppm)
- : not done or not applicable

proposed structure of impurity A (or its salt)



Isatinoxim



All studies were conducted using a test batch characterized analytically, i.e.:

- Batch 5059972 was used for all studies performed in 1993-1996 (ref. 1-5; 7-12)
- Batch 503-3427 was used for the *in vitro* percutaneous absorption study, performed in 2002 (ref. 14)
- Batch 030031P050 was used for studies conducted in 2004 (ref. 6, 13)
- Batch 3381-198 was used in studies involving radio-labelled Isatin (ref. 13, 14)

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. "Purity, composition and substance codes".

3.1.6. Solubility

According to a certificate in ref. 20 (p. 25)

Water	:	1.1 g/l (OECD Method A6)
Ethanol	:	1 g/100 ml
DMF	:	1 g/100 ml

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow} : 0.70

3.1.8. Additional physical and chemical specifications

Organoleptic properties	:	/
Melting point	:	201 °C
Boiling point	:	/
Flash point	:	/
Vapour pressure	:	/
Density	:	/
Viscosity	:	/
pKa	:	/
Refractive index	:	/

Homogeneity
Isatin test material in PEG 300 (32 mg/ml) is demonstrated to be homogenous.

Stability
Isatin solutions (32 mg/ml in PEG and up to 250 mg/ml in DMF) are stable up to 4 h (investigation period) at room temperature, when protected from light and under inert gas atmosphere.

General comments on analytical and physico-chemical characterisation

The following properties do not or poorly comply with the basic requirements for proper characterisation:

* No data is presented on the stability of Isatin in marketed formulations.
* Isatin is not listed in the EU inventory of cosmetic ingredients.

3.2. Function and uses

Isatin is used in direct or oxidative hair colouring products at final concentrations up to 1.6%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline	:	OECD 401 (1987)
Species/strain	:	Rat, Crl:CD (SD)BR strain (VAF plus)
Group size	:	5 male + 5 female
Test substance	:	Isatin in polyethyleneglycol 300 solution
Batch no	:	5059972 (purity not stated)
Dose	:	2000 mg/kg bw in a volume of 10 ml/kg
Observ. period	:	14 days
GLP	:	Quality Assurance statement included

Groups of 5 male and 5 female rats received a single dose of test substance by gastric gavage. The animals were observed twice a day for mortalities and at regular intervals for clinical signs on the day of dosing and thereafter daily for 14 days. Bodyweights were recorded weekly and macroscopic abnormalities were recorded at autopsy. No histological examinations were performed.

Results
There were three mortalities: one male and one female were killed in extremis approximately 5.5h after dosing, and a second male 24h after dosing. Clinical signs in the animals prematurely sacrificed were hypoactivity, laboured respiration, lateral recumbency, piloerection and pale extremes. In surviving animals one female showed laboured respiration 1h after dosing and the

majority of animals showed hypoactivity on the day of dosing, but were normal thereafter. Weight gain was normal for the strain used. Autopsy of the animals surviving to day 14 revealed no treatment-related abnormalities. The oral LD50 was higher than 2000 mg/kg bw.

Ref.: 1

3.3.1.2. Acute dermal toxicity

/

3.3.1.3. Acute inhalation toxicity

/

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline	:	OECD 404
Species/strain	:	New Zealand albino rabbits
Group size	:	3 females
Test substance	:	Isatin, moistened with water
Batch no	:	5059972 (99.1% pure)
Dose	:	500 mg
GLP	:	Quality Assurance statement included

The day before dosing, the application site of test animals (about 6 cm² in dorsal area) was carefully clipped free of hair. A 0.5 g aliquot of Isatin, test batch 505 99 72 (99.1% pure), was placed undiluted, moistened with water, over a gauze pad that was then applied to the skin of the animal and held in position by means of an elastic adhesive tape.
After 4 hours, the semi-occlusive dressing was removed, the application site gently cleaned and the potential skin reactions were assessed 1, 24, 48 and 72 hours afterwards.

Results
Slight orange/yellow staining was noted in 2 of the 3 rabbits at all observation times. In the third animal the staining prevented evaluation at the 1 hour observation point, but only persisted to 24 hours. No skin reactions were observed. Isatin tested undiluted was non-irritant to rabbit skin.

Ref.: 2

3.3.2.2. Mucous membrane irritation

Guideline	:	OECD 405
Species/strain	:	New Zealand albino rabbits
Group size	:	3 female
Test substance	:	Isatin, 10% aqueous solution
Batch no	:	5059972 (99.1% pure)
Dose	:	0.1ml
GLP	:	Quality Assurance statement included

A 0.1ml aliquot of a solution of Isatin 10% in water (w/v) was instilled in the conjunctival sac of the right eye of test animals. The potential ocular reactions were assessed 1, 24, 48 and 72 hours after instillation.

Results
There were no ocular reactions. The only findings were some orange staining up to 24 hours that did not preclude accurate assessment of ocular reactions. Isatin at 10% in water (w/v) was non-irritant to rabbit eye.

Ref.: 3

3.3.3. Skin sensitisation

Guinea Pig Maximisation (Magnusson and Kligman)

Guideline	:	OECD 406
Species/strain	:	Dunkin-Hartley albino guinea pigs
Group size	:	20 female treated; 10 female controls
Test substance	:	Isatin
Batch no	:	5059972 (99.1% pure)
Dose	:	Induction: intradermal 0.1% in propylene glycol; epicutaneously 25% in propylene glycol Challenge: 25% in propylene glycol
GLP	:	Quality Assurance statement included

The concentrations used were selected based on separate ranging studies. These preliminary studies showed that 0.1% (w/v) Isatin in propylene glycol was the maximal concentration eliciting acceptable irritant response following intradermal injection, and that 25% (w/v) Isatin in propylene glycol was the maximal practicable concentration. As the latter concentration was non-irritant, the topical induction application of Isatin was preceded by the topical application of 10% Sodium Lauryl Sulphate in liquid paraffin. The application sites were clipped free of fur before each treatment. The induction procedure consisted of intradermal injections and topical application, 7-day apart.

On Day 1, the animals received 3 pairs of intradermal injections in the dorsal area between the shoulders. The 3 pairs of injections consisted of:

- 50% (v/v) FCA in water for both control and treated animals
- 0% or 0.1% (w/v) of Isatin in propylene glycol for control and treated animals, respectively
- Isatin at 0.1% (w/v) or propylene glycol in FCA/water (50/50, v/v) for treated or control animals, respectively.

On Day 8, patches of filter paper (about 8 cm^2) saturated with 0 or 25% (w/v) Isatin in propylene glycol were applied over the injection sites of control and treated animals, respectively, and held occlusively for 48 hours by means of surgical tape and elastic adhesive bandage (topical induction).

Fourteen days after the topical induction (Day 22), patches of filter paper saturated with 0 or 25% (w/v) Isatin in propylene glycol were applied for all control and treated animals over the

right and left flank, respectively (challenge topical application). Patches were held occlusively for 24 hours as previously described. The animals were examined and cutaneous reactions assessed 24 and 48 hours after dressing removal.

Results
Examinations at 24 hours were made difficult due to the orange staining elicited by Isatin. However, at 48 hours, 20/20 (100%) treated animals showed a positive response to the challenge application, whereas none of the control animals (0/10) displayed such a reaction (24-hour and 48-hour observations).
Under the conditions of the present study, Isatin was considered to be a extremely potent skin sensitizer.

Ref.: 4

Guinea Pig (Buehler)

Guideline	:	OECD 406
Species/strain	:	Dunkin-Hartley albino guinea pigs
Group size	:	20 female treated; 10 female controls
Test substance	:	Isatin
Batch no	:	5059972 (99.1% pure)
Dose	:	Induction: 3 x 25% test substance. Challenge: 25% and 10%
GLP	:	Quality Assurance statement included

The concentration used was selected based on a dose-ranging study showing that 25% (w/v) Isatin in propylene glycol being the maximal practicable concentration.

Treated sites of test animals were clipped free of fur before each induction or challenge application. The induction procedure consisted of 3 weekly topical applications of Isatin.
On Day 1, a gauze pad (about 4 cm^2) saturated with 0 or 25% (w/v) Isatin in propylene glycol was applied over the left shoulder region of control or treated animals, respectively. It was held in place for 6 hours by an occlusive dressing. This treatment procedure was repeated on Days 8 and 15 of the study.

On Day 29, after a 2-week rest period, all control and treated animals received a topical challenge application of 10% and 25% Isatin in propylene glycol on right and left flank, respectively, as previously described. The animals were examined and cutaneous reactions assessed 24 and 48 hours after dressing removal.

Results
The test article caused orange/red staining of the application sites, which made assessment of erythema difficult. No positive responses were reported in any of the animals. The authors concluded that the substance showed no evidence of inducing contact allergy but the study is regarded as inconclusive because of the staining.

Ref.: 5

Local Lymph Node Assay

Guideline	:	OECD 429
Species/strain	:	CBA/J mice
Group size	:	56 female CBA/J mice were used in the main study, separated in two independent experiments conducted on 7 groups of 4 animals each
Test substance	:	Isatin
Batch no	:	030031P050 (100.4% pure)
Dose	:	0.1% to 25%
GLP	:	Quality Assurance statement included

The 7 groups used in each of 2 separate experiments consisted of:

- 5 treated groups tested at the chosen concentrations of Isatin in dimethylformamide (DMF). This vehicle was selected in a previous solubility study showing that 25% (w/v) Isatin was the maximal practicable concentration. In the first experiment, the concentrations used were 1, 2.5, 5, 10 and 25%, as this maximal concentration was not irritant in a preliminary test.
- A negative control group receiving the vehicle (DMF)
- A positive control group receiving alpha-hexylcinnamaldehyde (HCA), at 25% (v/v) in DMF

As positive results were obtained in the first experiment, a second experiment was performed at the concentrations of 0.1, 0.25, 0.5, 1 and 2.5%. These test concentrations were selected based on the results of the first experiment to determine more accurately the EC_3 value of Isatin.
In each experiment, Isatin, DMF or HCA was applied over the ears (25 µL per ear) for three consecutive days (designated as Days 1, 2 and 3). After 2 days of resting, the proliferation of the lymph node cells in the lymph nodes draining the application sites was measured by incorporation of tritiated methyl thymidine (^{3}H-TdR, Day 6). The values achieved were used to calculate stimulation indices (SI), and the EC_3 was estimated (concentration resulting in a SI of 3). The irritant potential of the test item was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
In the first experiment performed at 1, 2.5, 5, 10 and 25%, SI values were above the threshold positive value of 3 at all tested concentrations (SI ranging from 5.3 at 1% to 15.6 at 10%). Therefore, in the absence of local irritation, these lymphoproliferative responses were attributed to delayed contact hypersensitivity. The EC_3 value of Isatin was lower than 1% in this first experiment.

In the second experiment performed at 0.1, 0.25, 0.5, 1 and 2.5%, SI values were close to 1 at all tested concentrations except at 2.5% where it was close to the threshold value of 3 (SI of 2.93 at 2.5%). The EC_3 value was considered to be close to 2.5% in this second experiment.
Even if lymphoproliferative responses attributed to delayed contact hypersensitivity were obtained in both experiments, differences in the determination of the EC_3 value were observed (EC_3<1% in first experiment, EC_3=2.5% in second experiment). This discrepancy could be explained by a low incorporation of ^{3}H-TdR in the vehicle control group of the first experiment (dpm/group = 268), leading to excessively high SI values. Indeed, even if this first experiment was considered valid, the incorporation of ^{3}H-TdR in the vehicle control group was close to the

lower limit of historical data (mean = 777; min-max = 257-1496). Therefore, the evaluation of the skin sensitization potential of Isatin was rather based on the results of the second experiment, for which a more usual value of incorporation of ^{3}H-TdR was observed in the vehicle control group (dpm/group = 576).

Under the conditions of this study, Isatin induced delayed contact hypersensitivity in the murine Local Lymph Node Assay. According to the EC_3 value estimated in this study (2.5%), Isatin was considered as a moderate sensitizer.

Ref.: 6

3.3.4. Dermal / percutaneous absorption

Guideline	:	OECD 428 (draft)
Tissue	:	human abdominal skin
Tissue integrity	:	transepidermal water loss measurement
Method	:	flow through diffusion cells (6 ml/h)
Test substance	:	ISATIN 1.6 % in oxidative formulation (formulation + H_2O_2 40 %) aqueous formulation without oxidation (formulation + 40 % water)
Dose applied	:	formulation 20 mg/cm², i.e. 320 µg of ISATIN
Assay	:	liquid scintillation counting 2,3-^{14}C-ISATIN
Contact	:	30 minutes, then washing of the skin surface, monitoring of the diffusion during 24 hours
Batch no	:	radioactive compound batch 3381-198, radiochemical purity 99 % by HPLC, non-labelled ISATIN batch 503.3427, purity 99.7 %.
Replicate cells	:	8 cells (4 donors) for each formulation
GLP	:	study in compliance

The skin penetration of ISATIN was evaluated in a flow through diffusion cell. Human abdominal skin was obtained from 4 different donors and dermatomed to a controlled thickness (656 ± 128 µm). The receptor fluid in the dermal compartment was a phosphate buffer containing 0.25 % of Tween 80 in order to maintain "sink" conditions (solubility of ISATIN in the medium 206 µl/ml). Under the experimental conditions the skin temperature was 32.6°C ± 0.1°C. The hair dye was applied (20 mg of formulation/cm² - 1.6% ISATIN (w/w)) over 2 cm² during 30 minutes, the skin was washed with 2% sodium lauryl sulphate solution and dried. The diffusion was monitored during 24 hours. The mass balance of the study was calculated (106.3 ± 2.1 % and 102.6 ± 7.1 %)

Results
The absorbed amounts were as follows (sum of amounts contained in epidermis, dermis and receptor fluid):

- oxidative formulation: 3.87 ± 1.70 $\mu g_{eq}/cm^2$ (1.26 ± 0.51% of the applied dose)
- aqueous formulation: 2.39 ± 0.84 $\mu g_{eq}/cm^2$ (0.72 ± 0.25% of the applied dose)

The dermal absorption of ISATIN contained at 1.6% in hair dye formulations was estimated to be at most 3.87 ± 1.70 $\mu g/cm^2$ (1.26 ± 0.51% of the applied dose). The study is acceptable.

Ref.: 14

Penetration in the presence of hydrogen peroxide

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	Isatin, 1.4% in formulation/H_2O_2 mixture
Batch no	:	5059972 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	12 cells (10 donors) with hair and 11 cells (11 donors) without hair
GLP	:	not in compliance

The skin penetration of Isatin was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen breast skin of a number of different donors. The test substance was prepared at a concentration of 1.4% in a formulation containing hydrogen peroxide. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane with and without addition of 10 mg finely chopped bleached hair for 30 minutes and then excess washed off with 2% sodium lauryl sulphate solution and dried. Four hours later, the levels of substance were measured in the receptor fluid (physiological saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study, and by means of addition of Chinese ink. Any cells showing penetration of the ink were eliminated from the analysis.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.13% of applied dose in the presence and absence of hair.
This study did not include determination of recovery of the test substance. Physiological saline was used as the receptor fluid, which may not be adequate for a relatively lipophilic substance and insufficient time was allowed for permeation from the epidermal membrane into the receptor fluid.
The study is considered inadequate (see SCCNFP note of guidance).

Ref.: 15

Penetration in the absence of hydrogen peroxide

Guideline	:	/
Tissue	:	Human abdominal epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	Isatin, 1.5% in formulation/H_2O_2 mixture
Batch no	:	5059972 (purity not stated in study report)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	20 cells (10 donors) with hair and 19 cells (11 donors) without hair
GLP	:	not in compliance

The skin penetration of Isatin was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen breast skin of a number of different donors. The test substance was prepared at a concentration of 1.5% in a formulation without hydrogen peroxide. Approximately 40 mg of the mixture was applied to 2cm^2 of epidermal membrane with and without addition of 10 mg finely chopped bleached hair for 30 minutes and then excess washed off with 2% sodium lauryl sulphate solution and dried. Four

hours later the levels of substance were measured in the receptor fluid (physiological saline) using HPLC. Integrity of the epidermal membrane was checked by microscopy before the study, and by means of addition of Chinese ink. Any cells showing penetration of the ink were eliminated from the analysis.

Results
The quantity of test substance penetrating through the epidermis to the receptor fluid corresponded to 0.19% of applied dose in the presence of hair and 0.20% of applied dose in the absence of hair.
This study did not include determination of recovery of the test substance. Physiological saline was used as the receptor fluid, which may not be adequate for a relatively lipophilic substance and insufficient time was allowed for permeation from the epidermal membrane into the receptor fluid.
The study is considered inadequate (see SCCNFP note of guidance).

Ref.: 15

3.3.5.	Repeated dose toxicity

3.3.5.1	Repeated dose (28 days) oral / dermal / inhalation toxicity

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

Guideline	:	OECD 408 (1981)
Species/strain	:	Crl: CD (SD) BR strain rat, (VAF plus)
Group size	:	10 males + 10 females
Test substance	:	Isatin in polyethyleneglycol 300
Batch no	:	5059972 (purity 99.1%)
Dose levels	:	0, 10, 50 and 250 mg/kg bw/day, 7 days/week by gavage
Exposure period	:	13 weeks
GLP	:	Quality Assurance statement included

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 0, 10, 50 and 250 mg/kg bw/day, 7 days/week for 13 weeks. The dosing solutions were analysed during weeks 1 and 13 for stability and verification of homogeneity and concentration. During the study, the animals were observed daily for clinical signs and mortality, and weekly for bodyweight and food consumption. During week 12, urine was collected overnight for urinalysis, and in week 13, blood was sampled from the lateral tail vein for haematology and blood biochemistry. At the end of the treatment periods, a full autopsy was conducted with recording of weights and macroscopic and microscopic examination of major organs. Opthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results
There were 3 mortalities: one control female killed in extremis on day 86 with a damaged tail, and 2 low dose males, one found dead on day 16 without prior symptoms and one killed in extremis on day 36. These were not considered to treatment-related. No treatment-related clinical signs of toxicity were reported. Hair loss and scabbing were noted in some animals of all

dose groups, including controls. All high dose animals showed generalised yellow staining of the fur from day 9, and orange peribuccal staining from day 16. These were considered to be due to the colour of the test substance and not of toxicological significance. Bodyweight gain and food consumption were similar for all dose groups. Ophthalmological examinations revealed no differences between control and treated animals.
There were apparent dose-related decreases in red blood cell count in both sexes and in packed cell volume in female rats, showing statistical significance from control in all treatment groups (changes within 10% of control). The author noted that the changes were within the range of historical controls at 10 and 50 mg/kg bw/day, but not at 250 mg/kg bw/day, and concluded that the changes were only treatment-related in the high dose groups. Reticulocytes were not recorded. Blood triglyceride and inorganic phosphate levels were significantly elevated in the high dose male rats, with some values above historical controls. Other minor changes in haematology and biochemistry were within the range of historical controls and not considered to be treatment-related. Interpretation of urinalysis was made difficult by contamination of urine with faeces in some animals of all dose groups. Urine volume was increased in high dose males. All other parameters were similar to control.

Absolute and relative heart weight and relative liver weight were elevated in the males treated at 250 mg/kg bw/day. The changes were within 10% of controls, were considered normal for the age and strain of rat and therefore not treatment-related. The macroscopic changes noted at autopsy related to the staining properties of the test substance. Minor abnormalities observed in the histopathological examination were within the normal range of background alterations and were not treatment-related.
The effects seen on haematology and biochemical parameters seen in male animals treated at 250 mg/kg bw/day were considered to be treatment-related and the NOAEL was 50 mg/kg bw/day.

Ref.: 5

3.3.5.3. Chronic (> 12 months) toxicity

/

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline	:	OECD 472 (1983)
Species/strain	:	*S. typhimurium*, TA98, TA100, TA1535, TA1537; *E. coli*, WP2uvrA
Replicates	:	Triplicate plates, two independent tests
Test substance	:	Isatin in DMSO solution
Batch no	:	505 99 72 (purity not stated in study report)
Concentrations	:	8-5000 µg/plate with and without metabolic activation
GLP	:	in compliance

Isatin has been investigated for gene mutation in *Salmonella typhimurium* and *Escherichia coli*. Liver S9 fraction from rats induced with β-naphthoflavone/phenobarbitone was used as the exogenous metabolic activation system. A preliminary study revealed no toxicity and therefore

the concentration range was based on the recommended maximum of 5000 µg/plate. Negative and positive controls were in accordance with the OECD guideline.

Results
No statistically significant increases in revertants were seen in any of the tester strains. The positive control agents gave the expected results.

Ref.: 8

In vitro chromosome aberration test in CHO cells

Guideline	:	OECD 473
Species/strain	:	Chinese Hamster Ovary Cells
Replicates	:	2 independent tests
Test substance	:	Isatin OBA
Batch no	:	505 99 72 (purity 99.1%)
Concentr. scored	:	103 - 229 µg/ml without metabolic activation
		300 - 729 µg/ml with metabolic activation
GLP	:	in compliance

Isatin OBA has been investigated for induction of chromosomal aberrations in CHO cells with a harvest time of 20 hours (continuous exposure without metabolic activation, 2h exposure with metabolic activation). Liver S9 fraction from Aroclor1254-induced was used as the exogenous metabolic activation system. Test concentrations for scoring were based upon a 50-80% reduction in mitotic index. Negative and positive controls were in accordance with the OECD guideline.

Results
In both experiments, most concentrations of test substance induced a significant increase in frequency of aberrations, both with and without metabolic activation.

Ref.: 9

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline	:	OECD 474
Species/strain	:	Mouse, CD1 outbred strain
Group size	:	5 males + 5 females
Test substance	:	Isatin in polyethyleneglycol 300
Batch no	:	505 99 72 (purity not stated in study report)
Dose levels	:	0, 125, 200 and 320 mg/kg bw; single dose by gavage
Sacrifice times	:	24, 48 and 72 hours
GLP	:	in compliance

Isatin has been investigated for induction of micronuclei in the bone marrow cells of mice. OECD guideline 474 is not cited in the report, but the study appears to correspond to its requirements. Preliminary toxicity studies showed mortalities at 500, 800 1250 and 2000 mg/kg

bw and therefore 320 mg/kg bw was used as the highest dose in the micronucleus assay. Negative and positive controls were in accordance with the OECD guideline.

Results
There were slight but statistically significant increases in the frequency of micronucleated erythrocytes at 24 hours in female mice treated at 125 mg/kg and at 72 hours in male mice treated with the test substance at 200 and 320 mg/kg. The relevant concurrent vehicle control groups exhibited very low incidence of micronuclei, compared with other control groups within the study. The author noted that the numbers of micronuclei in all treated groups of mice were within the normal range for the laboratory and considered that the increases were not of biological significance. There were no changes in the ratio of normal to polychromatic erythrocytes. The positive control agent gave the expected result.
The study was conducted adequately and did not give evidence of mutagenicity under the test conditions used. There was no cytotoxic effect on bone marrow cells directly indicating exposure of the target cells. However, toxicokinetic data suggest systemic availability of Isatin.

Ref.: 10

Rat liver *in vivo/in vitro* UDS assay

Guideline	:	draft OECD guideline (1991)
Species/strain	:	Wistar rat, HanIbm: WST (SPF) strain
Group size	:	3 males
Test substance	:	Isatin in polyethyleneglycol 300
Batch no	:	5059972 (purity 99.1%)
Dose levels	:	0, 150 and 1500 mg/kg bw, by gavage
Sacrifice times	:	16 hours: all dose groups; 2h: high dose group
GLP	:	in compliance

Isatin has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. A preliminary toxicity study resulted in clinical signs of toxicity but no mortalities at 1500, 1800 and 2000 mg/kg bw, and 1500 mg/kg bw was selected as the upper dose for the UDS study. Negative and positive controls were in accordance with the OECD guideline. Animals were sacrificed after 16 hours, and for an additional high dose group after 2 hours. Four animals were dosed per group, and three of them used for isolation of hepatocytes, which were then treated with ^{3}H-thymidine *in vitro*. Incorporation of radiolabel was assessed using autoradiography.

Results
One of eight rats dosed at 1500 mg/kg bw died and remaining animals exhibited piloerection and apathy after dosing. There were no differences in the viability of hepatocytes isolated from rats of different dose groups. The results met all the pre-defined criteria for a negative response and therefore the test substance was not found to induce UDS. The positive control agent gave the expected results.

Ref.: 11

3.3.7. Carcinogenicity

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3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

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3.3.8.2. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rat, Crl: CD (SD) BR (VAF plus) strain
Group size	:	24 females (mated)
Test substance	:	Isatin in polyethyleneglycol 300
Batch no	:	505 99 72 (purity 99.1%)
Dose levels	:	0, 50, 150 and 500 mg/kg bw/day
Treatment period	:	Days 6 to 15 of pregnancy, inclusive
GLP	:	in compliance

Groups of 24 female rats were dosed with the test substance by gavage at 0, 50, 150 and 500 mg/kg bw/day on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality, bodyweight was recorded on days 0, 6, 9, 12, 15 and 20 and food consumption on days 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). The concentrations, homogeneity and stability of the dosing formulations were verified analytically.

Results

Two females were found dead: a control animal on day 8, and a low dose animal on day 7. These deaths were attributed to dosing errors and there were no other mortalities. There were no treatment-related clinical signs except for post-dose salivation in all animals during the final 3-5 days of dosing at 500 mg/kg bw/day. Hair loss was reported in some animals of all dose groups. The high dose group animals exhibited reduced weight gain compared to controls at the start of the dosing period (days 6-9) and actual mean body weight was lower than controls throughout the dosing and post-dosing period. The difference was statistically significant on days 6-9. Food consumption of the high dose group appeared slightly lower than for controls at the onset of dosing (days 6-9). The bodyweight gain and food consumption of animals dosed at 50 and 150 mg/kg bw/day did not differ from control.

A small number of macroscopic abnormalities were observed at the scheduled autopsy. These were not treatment-related and were not seen in the high dose animals. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses, sex distribution and the mean foetal bodyweights were similar for control and treated groups.

The incidence of major skeletal and external/visceral abnormalities was 0, 0, 0, and 4 at 0, 50, 150 and 500 mg/kg bw/day, and the occurrence of relatively rare and severe abnormalities indicated a treatment-related effect. The incidences of minor external and visceral abnormalities were in the normal range. The overall incidence of minor skeletal abnormalities was higher than background in all groups, including control, and was not dose-related. However, some minor

abnormalities (e.g. abnormal vertebrae, extra ribs, degree of ossification of the thoracic centra) were more frequent in the high dose group foetuses. Abnormalities seen in the foetuses of dams treated with 50 and 150 mg/kg bw/day were within the normal range and were not considered to be treatment-related.
The test substance elicited maternal toxicity and developmental toxicity at 500 mg/kg bw/day. The NOAEL was 150 mg/kg bw/day.

Ref.: 12

3.3.9. Toxicokinetics

Guideline	:	/
Animal	:	CD1 mice (7 weeks)
Method	:	administration by gavage
Test substance	:	ISATIN 10 ml/kg in PEG 300
Dose	:	320 mg/kg
Assay	:	liquid scintillation counting 2,3-^{14}C-ISATIN
Sampling	:	3 animals/sex/time point: 0.5 – 1 – 2 – 3 – 4 – 8 – 24 – 48 hours
Batch no	:	radioactive compound batch 3381-198, radiochemical purity 99 % by HPLC, non labelled ISATIN batch 030031P050 purity 100.4 %.
GLP	:	in compliance

The plasma profile of radioactivity was determined in CD1 mice (24/sex) given a single oral gavage of [^{14}C]-Isatin at 320 mg/kg in 10mL/kg Polyethylene Glycol 300 (PEG 300). The experimental conditions used (animal species, strain, age, dose-level, route, vehicle and dosage volume) were similar to those used in a previous mouse bone marrow micronucleus test (10). Blood samples were collected from the animals at the following time points: 0.5, 1, 2, 3, 4, 8, 24 and 48 hours after dosing (3 mice/sex/time point, each mouse bled once). The plasma was analysed for total radioactivity by Liquid Scintillation Counting.
Mean plasma total radioactivity levels increased quickly over time to a first maximum, C_{max1}, at 1 hour (males: 104 μg_{eq}/ml) or 0.5 hours after dosing (females: 141 μg_{eq}/ml), followed by a decrease and then a rise to a second maximum, C_{max2}, at 4 hours (males: 107 μg_{eq}/ml) or 3 hours after dosing (females: 142 μg_{eq}/ml). Thereafter, plasma radioactivity levels decreased steadily until the last quantifiable time point at 24 hours for males (3.45 μg_{eq}/ml) or 8 hours (9.56 μg_{eq}/ml) for females.

CD1 mice given a single oral (gavage) dose of [^{14}C]-Isatin at 320 mg/kg in PEG 300 (10 ml/kg) were extensively exposed to Isatin-related material, with mean values of systemic exposure ($AUC_{0-\infty}$) of 739 μg_{eq}.h/ml and 605 μg_{eq}.h/ml for males and females, respectively. The terminal half-life, $t_{1/2z}$, was 4.43 hours for males and 1.24 hour for females. The plasma radioactivity profiles were characterized by two peaks, indicative of enterohepatic recirculation. All data was characterized by a low inter-animal variability, and there were no gender differences in pharmacokinetic parameters.

Ref: 13

3.3.10. Photo-induced toxicity

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3.3.11. Human data

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3.3.12. Special investigations

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3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**3.87 µg/cm^2**
Typical body weight of human		**=**	**60 kg**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.71 mg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.045 mg/kg**
No observed effect level (mg/kg) (rat, subchronic, oral)	**NOAEL**	**=**	**50 mg/kg**

Margin of Safety	**NOAEL / SED**	**=**	**1111**

3.3.14. Discussion

The overall package of tests is adequate and most have been conducted to GLP and appropriate guidelines. The acute oral toxicity in rats was low. A 13-week oral repeated dose study in rats showed changes in haematological and biochemical parameters at 250 mg/kg bw/day and a NOAEL of 50 mg/kg bw/day was set based on a sub-chronic toxicity study.
Maternal and developmental toxicity were reported following administration at 500 mg/kg bw/day during the stages of organogenesis. The NOAEL was 150 mg/kg bw/day.

It was non-irritating to rabbit skin when applied neat. It was non-irritating to the rabbit eye at a concentration of 10% in aqueous solution, a concentration reported to be insoluble according to the COLIPA summary. The sensitisation studies demonstrate that the substance is a sensitiser.

The percutaneous penetration of the test substance at 1.6% in hair dye formulations was estimated to be $\leq 3.87 \pm 1.70$ µg/cm^2 (1.26 ± 0.51% of the applied dose).

The substance induced chromosomal aberrations in mammalian cells *in vitro* but no bacterial gene mutations. Two *in vivo* genotoxicity studies using complementary species and endpoints indicated that the *in vitro* mutagenic potential was not expressed *in vivo*.

4. CONCLUSION

4.1. Oxidative hair dye formulations

The SCCP is of the opinion that the information submitted is inadequate to assess the safe use of the substance.

Before any further consideration, the following information is required:

* stability in cosmetic hair dye formulations;

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

4.2. Direct hair dye formulations

The SCCP is of the opinion that the use of Isatin as hair colouring agent ('direct' dye) in semi-permanent hair dye formulations at a maximum concentration of 1.6% in the finished cosmetic product does not pose a risk to the health of the consumer.

This hair dye, like many other hair dyes, is a skin sensitiser.

5. MINORITY OPINION

Not applicable

6. REFERENCES

The references in italics were not submitted as full reports. They consist of reports for studies considered inadequate (15), reports for range finding toxicity studies (17, 19) or publications (19).

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7. ACKNOWLEDGEMENTS

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Prof. R. Dubakiene
Prof. C.L. Galli
Prof. V. Kapoulas
Prof. N. Loprieno
Prof. J.-P. Marty
Prof. T. Platzek (Chairman)
Dr. S.C. Rastogi
Prof. T. Sanner
Prof. G. Speit (Rapporteur)
Dr. I.R. White