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

CONCERNING

LAWSONE

COLIPA n° C146

adopted by the SCCNFP during the 21st plenary meeting
of 17 September 2002

Foreword

The SCCNFP was requested to evaluate the additional genotoxicity data and the in vitro percutaneous absorption study enclosed in submission III and to inform the Commission whether these new results justify a modification of the opinions on Lawsone adopted by the SCCNFP during the 16th plenary meeting of 13 March 2001 and during its 19th plenary meeting of 27 February 2002.

This opinion replaces the SCCNFP opinions on lawsone adopted on 12 March 2001 (doc. n° SCCNFP/0385/00, final) and of 27 February 2002 (doc. n° SCCNFP/0561/02, final).

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 Lawsone safe for use as a non-oxidising colouring agent for hair dyeing?

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

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

Lawsone (INCI name)

2.1.2. Synonyms

2-hydroxy-1,4-naphthoquinone
1,4-naphthalenedione, 2-hydroxy-
1,4-naphthoquinone, 2-hydroxy-
2-hydroxy-1,4-naphthalenedione
2-hydroxynaphthoquinone
CI Natural Orange 6

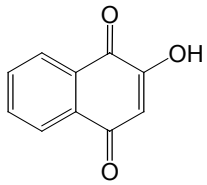
2.1.3. Trade names and abbreviations

Trade name : not stated
COLIPA n° : C146
Colour Index Number : CI 75480

2.1.4. CAS no.

CAS no : 83-72-7

2.1.5. Structural formula



2.1.6. Empirical formula

Emp. Formula : $C_{10}H_6O_3$
Mol weight : 174.16

2.1.7. Purity, composition and substance codes

All analytical data relate to batch 8160:FE.

Purity

Titre as determined by potentiometry	:	99.7%
Water content	:	0.4%
Ash content	:	< 0.2%
Heavy metals	:	< 10 ppm

Potential impurities

Reagents and intermediate reaction products

1,4-naphthoquinone	:	< 100 ppm
2-hydroxy-1,4-naphthoquinone-3,3'-dimer	:	0.264%
Acetic acid 2,4-diacetoxy-1,4-dihydro-naphthalene-1-yl ester	:	< 500 ppm

Solvent residues

None detected (i.e. solvents such as methanol, ethanol, isopropanol, n-propanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene < 100 ppm)

2.1.8. Physical properties

Subst. Code	:	/
Appearance	:	Yellow to mustard coloured powder
Melting point	:	194.5°C
Boiling point	:	no information
Density	:	no information
Rel. vap. dens.	:	no information
Vapour Press.	:	no information
Log P_{ow}	:	no information

2.1.9. Solubility

Insoluble in water at 0.2%
Soluble in 95% ethanol at 0.5%
Soluble in methanol at 1% (50°C)
Soluble in ethyl glycol at 5% (80°C)
Soluble in dimethyl formamide at 5%

2.2. Function and uses

Lawsone is proposed to be used as a non-oxidising hair colouring agent at a maximum concentration of 1.5% (typical concentration 1.26%) in the finished cosmetic product.

TOXICOLOGICAL CHARACTERISATION

2.3. Toxicity

2.3.1. Acute oral toxicity

Rat

Guideline	:	Directive 92/69/EEC
Species/strain	:	Sprague Dawley rat ICO: OFASD (IOPS Caw) -strain
Group size	:	5 male + 5 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone suspended in 0.5% aqueous methylcellulose
Batch no	:	8160:FE (purity 99.4%)
Dose	:	200, 310, 500, 800, 1300 and 2000 mg/kg bw (females), 500 and 2000 mg/kg bw (males).
Observ. period	:	14 days
GLP	:	Quality Assurance statement included

Groups of 5 male and 5 female rats received a dose of test substance by gastric gavage. The animals were observed for mortalities and clinical signs for 14 days. Bodyweights were recorded at intervals and macroscopic abnormalities were recorded at autopsy.

Results
In females, 0, 1, 2, 3, 5 and 5 of 5 animals died at doses of 200, 310, 500, 800, 1300 and 2000 mg/kg bw, respectively. In the male dose groups, there were no deaths at 500 mg/kg, and 5/5 animals died at 2000 mg/kg. Deaths mainly occurred within 30 min of dosing. Clinical signs of toxicity were hypo-activity (at 200 mg/kg), piloerection, hyper-salivation and respiratory difficulties. Surviving animals recovered by 2 days (females) or 4 days (males). No abnormalities were seen in animals found dead or at scheduled autopsy.
The LD50 was calculated to be 570 mg/kg for female rats and between 500 and 2000 mg/kg for male rats.

Ref. : 1

2.3.2. Sub-chronic oral toxicity

First study

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague Dawley CD strain rat
Group size	:	10 male + 10 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone suspended in Arachis oil
Batch no	:	60522-32 (purity 99.9%)
Dose levels	:	0, 8, 20 and 50 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, 8, 20 and 50 mg/kg bw/day, 7 days/week by gavage for 13 weeks. The dosing solutions were analysed

before the start of the study for stability, and on each formulation (prepared weekly) for verification of concentration. During the study, the animals were observed for clinical signs and mortality (daily for 29 days and then weekly), weekly for bodyweight and food consumption, and during weeks 1, 6 and 12 for water consumption. 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. Ophthalmological examination was conducted before the start of the study and at the end of the treatment period on controls and high dose animals.

Results

There were no mortalities, except that one female (dosed at 20 mg/kg/day) was killed in extremis on day 53 following a physical injury. Coloration of the urine was noted in all treated animals with a dose-related intensity, accompanied by staining of the fur and tail at the high dose. Some high dose animals exhibited increased salivation towards the end of the study. Other clinical signs were sporadic throughout all dose groups and not considered to be treatment-related.

A slight decrease in food consumption was noted for the high dose animals of both sexes, during weeks 2 to 5. Consumption at other times and for other dose groups was comparable to control.

Bodyweight gain was decreased in males at 50 mg/kg bw/day, and in females throughout the dose groups in a clear dose-dependent manner throughout the study. Statistical analysis is not noted in the report. Terminal body weights were 98%, 93% and 90% of control at 8, 20 and 50 mg/kg bw/day, respectively. Water consumption was increased in high dose animals of both sexes during weeks 6 and 12. No abnormalities were noted during ophthalmological examinations.

There was evidence of haemolytic anaemia in females dosed at 50 mg/kg bw/day, seen as decreased haemoglobin, haemotocrit and erythrocyte counts, and increased mean corpuscular volume. These parameters were not significantly different from control in the males or lower dose females. All female dose groups exhibited a significant, but not dose-related decrease in clotting time.

Dose-related decreases in blood urea, creatinine and albumin/globulin ratio, and increased bilirubin were seen in females, which were significant at 20 and 50 mg/kg bw/day. Similar changes were seen in the males at the high dose, but not at the mid dose. Other slight differences in biochemical parameters were minor, not-dose related and not considered to be of toxicological significance.

Macroscopic abnormalities noted at autopsy related to the adrenals, bladder, forestomach and kidneys at 50 mg/kg bw/day, and confined to the stomach and kidney at 8 and 20 mg/kg bw/day.

Dose-related increases in kidney, liver and spleen weights were apparent for both sexes. These were significantly higher than control at all doses for the relative kidney weights in the male (112%, 118%, 124%, respectively), and for the relative liver weight in the females (108%, 114% and 131%, respectively). Relative spleen weights were significantly elevated in the mid and high dose animals (males: 123% and 161%; females: 128% 188%, respectively). These changes in weight were accompanied by a number of histo-pathological abnormalities. Extramedullary haemopoiesis and haemosiderin accumulation were noted in the spleen of both sexes at high dose and mid dose males. Renal tubular pigment deposits (Perl's positive) and tubular basophilia/dilatation/degeneration for both sexes at high dose. Acanthosis, hyperkeratosis and subepithelial inflammatory cell infiltrates were noted in the stomachs of animals of all dose groups.

The study failed to identify a NOAEL for gastric and renal effects.

Ref. : 5.1

Second study

Guideline	:	OECD 408 (1981)
Species/strain	:	Sprague Dawley Crl CD (SD)BR strain rat
Group size	:	10 male + 10 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone suspended in 0.5% aqueous methylcellulose
Batch no	:	8160:FE (purity 99.4%)
Dose levels	:	0, 2, 7 and 20 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, 2, 7 and 20 mg/kg bw/day, 7 days/week for 13 weeks. The dosing solutions were analysed before the start of the study for stability, and on each formulation (prepared weekly) for verification of concentration. During the study, the animals were observed daily for clinical signs and mortality, and weekly for bodyweight and food consumption. In week 13, overnight urine was collected and blood was sampled from the orbital sinus for urinalysis, 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. Ophthalmological examination was conducted before the start of the study and at the end of the treatment period on controls and high dose animals.

Results

There were no mortalities. Coloration of the urine was noted from week 10 in animals dosed at 20 mg/kg bw/day, accompanied by staining of the tail in females. Hyper-salivation was reported in 4/10 high dose males from week 2 onwards. Other clinical signs were sporadic throughout all dose groups and not considered to be treatment-related. Bodyweight gain and food consumption was comparable for all dose groups. A small number of minor abnormalities was reported from the ophthalmological examinations, with similar incidence in controls and treated animals.

There was an apparent dose-related decrease in erythrocyte count in females, which was significantly lower than control at 7 and 20 mg/kg bw/day. A significant decrease was also seen in males at the top dose only. Other small differences showed no evidence of dose-response relationship. The study authors considered that for all haematological parameters the differences were small and the individual values were within or close to the normal range, and concluded that they were not of toxicological significance.

Dose-related decreases in blood urea, creatinine and albumin/globulin ratio, and increased bilirubin were seen in females, for which albumin/globulin was significant at all dose levels, urea at 7 and 20 mg/kg bw/day, and creatinine only at the high dose. Creatinine was also significantly reduced in the males at the high dose. A dose-related decrease in blood glucose and increase in triglycerides was also apparent in the males. Other slight differences in biochemical parameters were not-dose related. The study authors considered that for all biochemical parameters the differences were small and the individual values were within or close to the normal range, and concluded that they were not of toxicological significance. There were no differences in urinary parameters.

Macroscopic abnormalities noted at autopsy related to the forestomach and kidneys in animals dosed at 20 mg/kg bw/day.

Dose-related increases in kidney and spleen weights were apparent for both sexes, and for liver of females. The female relative kidney weights were significantly higher than control at 7 and

20 mg/kg bw/day (115% and 129%, respectively). Other increases were only significant at the high dose (spleen: male 129%, female 128%; kidney: male 112%; liver: female 111%).
Minimal to slight haemopoiesis was noted in the spleen of some animals of all dose groups with an increased incidence and/or intensity in males at 7 mg/kg bw/day and in both sexes at 20 mg/kg bw/day.
Renal tubular basophilia was reported in some animals of all dose groups with an increased incidence and/or intensity in both sexes at 20 mg/kg bw/day, at which dose it was accompanied by dilatation and/or pigment accumulation degeneration in some animals. The incidence at 7 mg/kg bw/day was comparable to control. In the forestomach, minimal to slight focal or multifocal ulceration of the mucosa, or minimal to slight interstitial oedema were reported with an increased incidence in both sexes at 7 mg/kg bw/day.

The authors concluded that treatment-related effects occurred at 20 mg/kg bw/day, affecting mainly the kidneys, forestomach and spleen, and that the NOAEL was 7 mg/kg bw/day. Significant changes were seen at 7 mg/kg bw/day which were consistent with the effects at 20 mg/kg bw/day in this study, and at higher doses in the first 13-week study (ref. 5.1). A NOAEL of 2 mg/kg bw/day should therefore be assumed.

Ref. : 5.2

2.4. Irritation & corrosivity

2.4.1. Irritation (skin)

Guideline	:	OECD 404 (1987)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 males
Test substance	:	2-Hydroxy-1,4-naphthoquinone, neat and suspended at 2% in 0.5% aqueous methylcellulose
Batch no	:	8160 FE (purity 99.4%)
Dose	:	0.5 g or 0.5 ml
GLP	:	QA statement included

The substance was applied neat (0.5 g) to the right flank and as a 2% suspension in 0.5% aqueous methylcellulose (0.5 ml) to the left flank. In both cases the substance was applied to 6cm^2 of intact skin, and covered by semi-occlusive patches for 4 hours. Cutaneous reactions were evaluated 1, 24, 48 and 72 hours after removal of the patches.

Results
Orange staining due to the dye interfered with evaluation of erythema. No oedema was observed. The substance could potentially have provoked slight to moderate, but not severe irritation.

Ref. : 3

2.4.2 . Irritation (mucous membranes)

Guideline	:	92/69/EEC (1992)
Species/strain	:	New Zealand albino rabbit
Group size	:	3 male
Test substance	:	2-Hydroxy-1,4-naphthoquinone, neat
Batch no	:	8160 FE (purity 99.4%)

Dose	:	100 mg
GLP	:	QA statement included

The test substance was applied neat to the left eye of 3 male rabbits, without rinsing. The right eye served as control and was untreated. Ocular reactions were recorded at 1 hour and 1 to 7 days after instillation.

Results
Slight to moderate conjunctival irritation was reported in all three animals up to day 6, with all recovering by day 7. Slight irridial irritation was noted in 2/3 rabbits on day 1, but had resolved by day 2. Slight corneal opacity was noted in 3/3 animals 1 and 24 hours after instillation and had resolved by day 4. The mean scores for 2 of the 3 animals did not reach the criteria values for irritation specified in 91/325/EEC, and the substance was therefore classified as non-irritant. Based upon the observed reactions, the substance should be regarded as irritant to the rabbit eye.

Ref. : 2

2.5. Sensitisation

Magnusson and Kligman study

Guideline	:	OECD 406 (1981)	
Species/strain	:	Dunkin-Hartley guinea pig	
Group size	:	10 male + 10 female in test group, 5 male + 5 female in control group	
Test substance	:	2-Hydroxy-1,4-naphthoquinone dissolved in liquid paraffin	
Batch no	:	294028 (purity not stated)	
Concentrations	:	intradermal induction :	0.1 ml 50% Freund's complete adjuvant (FCA)
			0.1 ml 1% test substance
			0.1 ml 1% test substance/FCA (1:1)
		induction of irritation :	0.5 ml of 10% sodium lauryl sulphate in vaseline
		topical induction :	0.5 ml 1% test substance for 48 hours, occluded
		challenge :	0.5 ml 1% test substance for 24 hours, occluded
GLP	:	Quality Assurance statement included	

Induction commenced with three intradermal injections, of FCA, test substance (1.0%), and a mixture of these two. Six days later 0.5 ml of 10% lauryl sulphate was applied to the injection site to induce a local irritation, and the next day the induction process was completed with a single topical application of 0.5ml of the test substance (1%) under occlusive patch for 48 hours. An interval of 2 weeks was allowed after induction and then the animals were challenged by a single 0.5 ml topical application of the test substance (1%) under occlusive patch on the flank for 24 hours. Appropriate controls were treated with vehicle. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results
After the challenge, evaluation of erythema was obscured by brown staining of the skin at both 24 and 48 hours. Oedema was not observed in any of the animals. Histological examination of skin biopsies revealed changes in all treated animals. The authors considered that the reactions in 13/20 animals were due to sensitisation reactions and classified the substance as a strong sensitiser. However, it is not possible to distinguish between irritation and sensitisation on the basis of histological examination.

Ref. : 4.1

Magnusson and Kligman study

Guideline	:	OECD 406 (1992)
Species/strain	:	Dunkin-Hartley guinea pig
Group size	:	10 male + 10 female in test group, 5 male + 5 female in control group
Test substance	:	2-Hydroxy-1,4-naphthoquinone dissolved in paraffin oil
Batch no	:	8160:FE (purity 99.4%)
Concentrations	:	intradermal induction : 0.1 ml 50% Freund's complete adjuvant (FCA) 0.1 ml 10% test substance 0.1 ml 10% test substance/FCA (1:1) induction of irritation : 0.5 ml of 10% sodium lauryl sulphate in vaseline topical induction : 0.5 ml 40% test substance for 48 hours, occluded challenge : 0.5 ml 40% and 2% test substance for 24 hours, occluded
GLP	:	Quality Assurance statement included

Induction commenced with three intradermal injections, of FCA, test substance (40%), and a mixture of these two. Six days later 0.5 ml of 10% lauryl sulphate was applied to the injection site to induce a local irritation, and the next day the induction process was completed with a single topical application of 0.5ml of the test substance (40%) under occlusive patch for 48 hours. An interval of 2 weeks was allowed after induction and then the animals were challenged by a single 0.5 ml topical application of the test substance (40%) on the left flank and 2% on the right flank under occlusive patch for 24 hours. Appropriate controls were treated with vehicle. The skin was examined 24 and 48 hours after removal of the challenge patches.

Results

After the challenge, evaluation of erythema was obscured by orange staining of the skin at both 24 and 48 hours. Oedema was not observed in any of the animals. Histological examination of skin biopsies revealed changes in all treated animals, which were considered to be equivocal by the study authors. However, the slides were peer-reviewed by CIT at a later date, resulting in the conclusion that sensitisation occurred in 8/10 animals challenged with 2% test substance and in 9/10 animals challenged with 40% test substance. Remaining animals exhibited reactions that did not meet the criteria either for a positive response or for a negative response and were therefore considered to be equivocal.

However, it is not possible to distinguish between irritation and sensitisation on the basis of histological examination.

Ref. : 4.2

2.6. Teratogenicity

Guideline	:	OECD 414 (1981)
Species/strain	:	Sprague-Dawley rat, Crl: CD (SD) BR strain
Group size	:	25 females (mated)
Test substance	:	2-Hydroxy-1,4-naphthoquinone suspended in 0.5% aqueous methylcellulose
Batch no	:	8160:FE (purity 99.4%)
Dose levels	:	0, 2, 7 and 20 mg/kg bw/day
Treatment period	:	Days 6 to 15 of pregnancy, inclusive
GLP	:	Quality Assurance statement included

Groups of 25 female rats were dosed with the test substance by gavage at 0, 2, 7 and 20 mg/kg bw/day on days 6 to 15 after mating. The dams were observed daily for clinical signs and mortality, bodyweight and food consumption were recorded on days 0, 2, 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 endpoint).

Results
There were no mortalities or clinical signs of toxicity. One high dose female aborted on day 15, which was not considered to be treatment-related because there were no prior signs of toxicity or macroscopic changes. Food consumption and bodyweight gains were significantly lower at 20 mg/kg bw/day but comparable for other dose groups. Mean bodyweight of the high dose group was 97% of control at the end of the treatment period (day 15). No treatment-related maternal abnormalities were noted at the scheduled autopsy. The mean numbers of corpora lutea, live foetuses, sex distribution and the mean foetal bodyweights were comparable for control and treated groups. The incidence of foetal abnormalities or malformations was comparable for all dose groups.
There was slight maternal toxicity at 20 mg/kg bw/day but not embryo-toxicity or teratogenicity. The NOAEL was 7 mg/kg bw/day for materno-toxicity.
The significance of the single abortion at 20 mg/kg bw/day is unclear. However it does not influence the conclusion with respect to the NOAEL.

Ref. : 12

2.7. Toxicokinetics (incl. Percutaneous Absorption)

2.7.1. Percutaneous Absorption *in vitro*

First Study

Guideline	:	none available
Tissue	:	Human mammary epidermis, heat-separated
Method	:	Franz diffusion cell (static)
Test substance	:	2-Hydroxy-1,4-naphthoquinone, 1.78% in formulation
Batch no	:	8160:FE (purity: 99.4%)
Dose levels	:	c. 40mg formulation in the presence/absence of 10 mg hair
Replicate cells	:	9 cells without hair and 15 cells with hair
GLP	:	Study not in compliance

The skin penetration of COLIPA C146 was evaluated in a static Franz diffusion cell system. Human epidermis was prepared by heat-separation from previously frozen mammary skin. The test substance was prepared at a concentration of 1.78% in a formulation. 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 at the end of the study.

Results:
Penetration was calculated to be 0.374% of applied dose in the presence of hair and 0.363% 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 Notes of Guidance).

Ref. : 13

Second study

An in vitro study on the percutaneous absorption into and through human skin of 2% Lawsone in a hair dye formulation is presented. The study is performed according to the standard protocol, described in the Notes of Guidance for Testing of Cosmetic Ingredients for their Safety Evaluation of 24.10.2000, Annex 10.

The testing procedure is appropriate for the assessment of the percutaneous absorption of this hair dye ingredient in view of the calculation of its margin of safety. The investigations were performed under GLP conditions. The presentation of the data is sufficiently detailed. The evaluation of the data, the calculations and the interpretation of the results are scientifically sound and correspond to the goal of the investigation.

A deviation from the standard procedure is noted :

20 mg/cm² of formulation were applied to the skin specimen, whereas 2 mg/cm² are recommended. Since however this represents an excessive amount, this deviation should have no consequences on the interpretation of the results, provided that as basis for safety assessment only the figure of amounts in µg/cm^2/24h is used.

The absorbed amounts of Lawsone are given as 2.6 ± 1.8 µg/cm². According to the protocol they can be interpreted as 2.6 ± 1.8 µg/cm²/24h.

Ref. : 35

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline	:	OECD 471 (1983)
Species/strain	:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2uvrA
Replicates	:	Triplicate plates, 2 independent tests
Test substance	:	2-Hydroxy-1,4-naphthoquinone in DMSO
Batch no	:	8161:FE (purity: 99.4%)
Concentrations	:	25 - 600 µg/plate with and without metabolic activation
GLP	:	Quality Assurance statement included

COLIPA C146 has been investigated for gene mutation in *Salmonella typhimurium* and *Escherichia coli* using a plate incorporation and pre-incubation protocol. Liver S9 fraction from rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system. The concentration range was selected following a preliminary study which showed toxicity at and above 500 μg/plate. Negative and positive controls were in accordance with the OECD guideline.

Results
There were no significant increases in revertants in any of the tester strains, with or without metabolic activation. The positive control agents gave the expected results.

Ref. : 6

Mammalian cell gene mutation assay

First study

OECD guideline	:	OECD 476 (1984)
Species/strain	:	Mouse lymphoma L5178Y $TK^{+/-}$ cells
Replicates	:	2 independent tests
Test substance	:	2-Hydroxy-1,4-naphthoquinone in DMSO
Batch no	:	294028 (purity not stated in study report)
Concentr. scored	:	25 – 800 μg/ml with and without metabolic activation
GLP	:	Quality Assurance statement included

COLIPA C146 has been investigated for induction of cell mutations at the TK locus in mouse lymphoma L5178Y cells. Liver S9 fraction from Aroclor 1254-induced rats was used as the exogenous metabolic activation system. The maximum concentration was determined on the basis of a preliminary study which showed toxicity at and above 500 μg/ml. Negative and positive controls were in accordance with the OECD guideline.

Results
Significant increases in mutation frequency were seen at all concentrations in the presence of S9 in both experiments, with a poor dose-response relationship. A slight increase was seen in the absence of S9. The substance showed mutagenic activity with and without metabolic activation.

Ref. : 7

Second Study

OECD guidelines	:	OECD 476 (1984)
Species/strain	:	V79 Chinese Hamster cell/ HPRT locus
Replicates	:	3 independent tests
Test Substance	:	2-HYDROXY-1,4-NAPHTHOQUINONE in DMSO
Batch No.	:	008160: FE (99.4% purity)
Concentr. scored	:	15-5000 μg/ml; 1000-5000 μg/ml; 500-5000 μg/ml (with and without met. act.)
GLP	:	OECD G.L.P. (1981)

COLIPA C146 has been investigated for induction of cell mutations at the HPRT locus in Chinese hamster V79 cells. Liver S9 fraction from Aroclor 1254 induced rats were used as the

exogenous metabolic activation system. The maximum concentration was determined on the basis of preliminary tests which showed some toxicity at 5000 µg/ml concentration.
Negative and positive controls were in accordance with the literature (MNNG; BaP). The updated version of the OECD Guideline indicates for this cell system (EMS; ENU; NMDA).

Results:
The mutation frequency in cells treated with COLIPA C 146 was higher than 3 times the vehicle control value for some concentrations (with and without metabolic activation).
Although this effect was reproduced in the different experiments, there was no dose-effect relationship. According to OECD this study provides equivocal results which should be clarified by further testing preferably using a modification of experimental conditions. (The 476 OECD Guideline has been updated in 1997. The study presented has been performed during 1996).

Ref. : 19

Mammalian cytogenetic assay in CHO cells

Guideline	:	OECD 473 (1983)
Species/strain	:	Chinese Hamster Ovary (CHO) cells
Replicates	:	Duplicate cultures, one experiment only
Test substance	:	2-Hydroxy-1,4-naphthoquinone in DMSO
Batch no	:	8160:FE (99.4%)
Concentr. scored	:	30, 100 and 300 µg/ml without metabolic activation 300, 1000 and 5000 µg/ml with metabolic activation
GLP	:	Quality Assurance statement included

COLIPA C146 has been investigated for induction of chromosomal aberrations in CHO cells. Liver S9 fraction from rats pre-treated with Aroclor 1254 was used as the exogenous metabolic activation system. The test concentrations were selected based on the recommended maximum according to current guidelines. Exposure was continuous without S9 and for 3 hours with S9, both with a 21 harvest time. Negative and positive controls were in accordance with the OECD guideline.

Results:
There was a significant increase in aberrant cell frequency, with activation at the top concentration of 5000 µg/ml (= 29 mmol concentration). There were no increases in the absence of S9. The positive control agent gave the expected result. The substance was clastogenic at 5000 µg/ml in the presence of metabolic activation. (The 473 OECD Guideline has been updated in 1997. The study presented has been performed during 1994).

Ref. : 8

2.8.2. Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test – first study

Guideline	:	OECD 474 (1983)
Species/strain	:	Mouse, Crl:NMRI BR outbred strain

Group size	:	5 male + 5 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone in DMSO
Batch no	:	batch not stated (purity: > 98%)
Dose levels	:	0 and 250 mg/kg bw, p.o.
Sacrifice times	:	24, 48 and 72 hours
GLP	:	Quality Assurance statement included

COLIPA C146 has been investigated for induction of micronuclei in the bone marrow cells of mice. The substance was administered once by gavage at 0 and 250 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:
There was a significant increase in the incidence of micro-nucleated polychromatic erythrocytes in the 72 hour test group (combined males and female data) but not at the other harvest times. The positive control agent gave the expected results.
The substance was positive in the micronucleus assay.

Ref. : 9.1

Mouse bone marrow micronucleus test – second study

Guideline	:	OECD 474 (1983)
Species/strain	:	Mouse, Crl:NMRI BR outbred strain
Group size	:	5 male + 5 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone in DMSO
Batch no	:	batch not stated (purity: > 98%)
Dose levels	:	0, 25, 110 and 250 mg/kg bw, p.o.
Sacrifice times	:	72 hours
GLP	:	Quality Assurance statement included

In a second study, the substance was administered once by gavage at 0, 25, 110 and 250 mg/kg bw and the bone marrow harvested after 72 hours. Negative and positive controls were in accordance with the OECD guideline.

Results:
There were significant increases in the incidence of micro-nucleated polychromatic erythrocytes at 110 and 250 mg/kg bw (combined males and female data) but not at 25 mg/kg bw. The results show evidence of a positive dose response relationship and were reported to be increased beyond the range of the historical negative control data. The positive control agent gave the expected results.
The study confirmed the results of the previous study.

Ref. : 9.2

Mouse bone marrow micronucleus test – third study

Guideline	:	OECD 474 (1983)
Species/strain	:	Mouse, Swiss OF1/ICO (IOPS Caw) strain
Group size	:	5 male + 5 female
Test substance	:	2-Hydroxy-1,4-naphthoquinone in 0.5% aqueous methylcellulose

Batch no	:	8160:FE (purity: 99.4%)
Dose levels	:	0, 30, 100 and 300 mg/kg bw, p.o.
Sacrifice times	:	24 and 48 hours
GLP	:	Quality Assurance statement included

In a third study, the substance was administered once by gavage at 0, 30, 100 and 300 mg/kg bw and the bone marrow harvested after 24 and 48 hours. Negative and positive controls were in accordance with the OECD guideline.

Results:
There were no significant differences between control and treated groups with respect to the incidence of micro-nucleated polychromatic erythrocytes at either harvest time. The ratio of polychromatic to normo-chromatic erythrocytes was significantly lower for all doses at the 24 hour harvest and at 30 and 300 mg/kg at the 48 hour harvest, indicating that the substance had reached the bone marrow. The positive control agent gave the expected results.
The authors concluded that the substance did not induce cytogenetic damage under the conditions of the assay. This study did not include the 72 hour harvest which showed positive results in the first two studies and is therefore considered inadequate.

Ref. : 9.3

Mouse bone marrow micronucleus test – fourth study

OECD guidelines	:	Not followed. This study has been performed according to Schimd (1975) and to Salamone et al. (1980). The study has been completed on July 6th 2001 at the CIT Laboratory France). The OECD 474 Guideline has been adopted on May 26th 1986 and updated on July 21st 1997.
Species/strain	:	Mice/Swiss/co:0F1 (IOPS Caw)
Group size	:	10 male mice treated; 5 male mice with vehicle (control)
Dose level	:	250 mg/kg (one oral treatment)
Test Substance	:	2-hydroxy-1,4-naphthoquinone CAS No. 83-72-7 (>98.5% HPLC) ACROS Organics, Belgium
Batch No.	:	A0147608
Negative control	:	0.5/ methylcellulose. Animals were sacrificed 72 hours after treatment
Positive control	:	absent
GLP	:	OECD GLP, 1997

The animals were treated orally and killed after 72 hours. For each animal hematology parameters were evaluated and the number of the micronucleated polychromatic erythrocytes (MPE) was counted in 2000 polychromatic erythrocytes. The PE/NE ratio was also evaluated.

Results :
Micronucleus : in the group of animals treated with COLIPA C146 the frequency of MPE as well as the PE/NE ratio was similar to that of the vehicle control group.
The study indicates that the chemical is non-toxic for the bone marrow cells of mice.

Hematology investigations :
In the animals treated with COLIPA C146 there were no treatment-related abnormalities, such as erythrocytes (RBC), haemoglobin (HB), mean cell volume (MCV), packed cell volume (PCV), mean cell haemoglobin concentration (MCHC). mean cell haemoglobin (MCH).

Conclusion :
The aim of this study was to evaluate the potential of the test substance to induce damage to the chromosome or to the mitotic apparatus in bone marrow cells of mice.
Under the experimental conditions, no conclusions can be drawn on the potential genotoxic effect of COLIPA C146 in vivo on mice, due to the absence of a positive control.

Ref. : 31

In vivo mammalian bone marrow cytogenetic test by oral route in mice chromosomal analysis – fifth study

Guideline	:	-
Species/strain	:	Swiss Ico: OF1 (IOPS caw)
Group size	:	10 male
Test substance	:	Lawsone suspended in 0.5 % aqueous methylcellulose.
Batch no	:	A014760801 (> 99 %)
Dose levels	:	0 and 250 mg/kg bw, p.o.
Sacrifice times	:	24 and 72 hours
GLP	:	Quality Assurance statement included

COLIPA C146 has been tested for induction of chromosome aberrations in vivo in the Swiss mice. The test substance was administered once by gavage at 0 and 200 mg/kg bw and the bone marrow harvested 24 and 72 hours. Negative and positive controls were in accordance with the OECD guideline.

Results :
Reactions to treatment : 3 animals died within few minutes following test item treatment and were replaced by supplementary animals. 2 additional animals died within 2 hours following administration.
Chromosomal aberrations : No statistically significant increase in the frequency of cells with structural chromosomal aberrations was observed in the treated group when compared to the vehicle control group at any harvest time (24 h & 72h). The positive control agent induced a dose-dependent increase of aberrant cells indicating the sensitivity of the test under the experimental conditions of the assay. The study is considered adequate.

Ref. : 33

Mouse bone marrow micronucleus test : Henna Rot (1992)

Guideline	:	OECD 474 (1983)
Species/strain	:	Albino CD-1 strain mice
Group size	:	5 male + 5 female
Test substance	:	Henna Rot (Lawsonia inermis) in 1% solution of carboxymethylcellulose
Batch no	:	830.72 (purity not given

Dose levels	:	0 and 300 mg/kg bw, p.o.
Sacrifice times	:	24, 48 and 72 hours
GLP	:	Quality Assurance statement included

Henna Rot has been investigated for its potential to induce micronuclei in bone marrow cells of mice.
A range finding study was conducted to determine the maximum tolerated dose level (MTD) and the most suitable route of administration (i.p. vs oral).
In the main study, the test substance was administered once by intragastric gavage at 0 and 300 mg/kg bw and 3 sacrifice times of 24, 48 and 72 hours after dosing were selected. Negative and positive controls were in accordance with the OECD guideline.

Results :
Reactions to treatment : In the main micronucleus test, no death occurred in any of the dose groups. The clinical signs observed were restricted to lethargy, ptosis, diarrhoea and emaciation.

NCE/PCE ratio : A significant increase has been observed in the ratio of NCE to PCE compared to the respective vehicle control for 48 h and 72 h sacrifice times. It should be noted that this increase is mainly due to the lowered number of mature cells (NCEs) ; this may reflect a toxic effects of this test agent on the bone marrow but does not supply information on the potential effects on the erythropoiesis.

Micronuclei : No statistically significant increase in the frequency of micronucleated PCE's was found in the treated group when compared to the vehicle control group at any sacrifice time (24 h, 48 h & 72h).
The positive control agent gave the expected results.
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 male and female mice after 24, 48 and 72 hours following a single oral administration of Henna Rot.

Remark :
The test substance Henna Rot (Lawsonia inermis) used in this study has a different name and COLIPA code (C 169) than the ones listed C 146 in the COLIPA summary.

Ref. : 37

28-day in vivo cytogenetic assay

OECD guidelines	:	does not exist
Literature guidelines	:	Unknown
Species/strain	:	Sprague-Dawley CD, male and female rats
Replicates	:	3 animals/sex/dose
Doses	:	vehicle control; 12.5 –25.0-50-100 mg/kg/day x 28 days
Genotoxic endpoint	:	chromosome aberrations in rat peripheral lymphocytes
Test Substance	:	2-hydroxy-1,4-naphthoquinone Lot 600522-32 of unknown purity
GLP	:	Internal Quality Ass. Unit.

COLIPA C146 was given daily orally to 4 groups of animals, one group of animals was treated with vehicle (arachis oil B.P.), one groups of animals was treated with Cyclophosphamide.

At the end of the treatment period (28 days) a sample of fresh blood was taken from the orbital sinus of each rat; the blood cultures were incubated for 48 hours and then treated with demicolcine. Slides prepared from the cultures were analysed for the presence of cytogenetic effects.
Data from only one group of COLIPA C146 treated animals are reported in the table, for the dose of 100 mg/kg/day.
No cytogenetic effect could be observed in the cultures derived from treated animals (vehicle=4.3%; C146=2.8%; CP=14.0%).
It is not possible to compare data obtained in this experiment with the data of literature, as the methodology employed has not been evaluated in general literature.

Ref. : 18

Chromosome aberration study in bone marrow cells of Chinese hamster

Guideline	:	-
Species/strain	:	Chinese hamster
Group size	:	5 male + 5 female
Test substance	:	FC 200488 suspended in arachis oil
Batch no	:	201 007/585 (purity not stated in study report)
Dose levels	:	0 and 200 mg/kg bw, p.o.
Sacrifice times	:	6, 24 and 48 hours
GLP	:	Quality Assurance statement included

COLIPA C146 has also been tested for induction of chromosome aberrations *in vivo* in the hamster. The substance was administered once by gavage at 0 and 200 mg/kg bw and the bone marrow harvested after 6, 24 and 48 hours. Negative and positive controls were in accordance with the OECD guideline.

Results:
There were no significant differences between control and treated groups with respect to the incidence of chromosome aberrations at any harvest time. The positive control agent gave the expected results. The substance did not induce cytogenetic damage under the conditions of the assay, which did not include the 72 hours analysis. The study is considered inadequate.

Ref. : 10

Rat liver *in vivo/in vitro* UDS assay

OECD guideline	:	draft guideline of 1991
Species/strain	:	Wistar rat, HanIbm: WST (SPF) strain
Group size	:	4 male
Test substance	:	FC 200488 in DMSO/polyethylene glycol 400 (1:9)
Batch no	:	29.6.93 (purity > 98%)
Dose levels	:	0, 150 and 1500 mg/kg bw
Sacrifice times	:	16 hours: all dose groups; 2h: high dose group
GLP	:	Quality Assurance statement included

COLIPA C146 has been investigated for induction of unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro* following *in vivo* dosing. A preliminary toxicity study resulted death of 2/2 animals at 2000 mg/kg bw and signs of toxicity but no mortalities occurred at 1500 mg/kg bw. 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:
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.

Remark:
The substance code (FC 200488) used in the last two studies is not listed in the synonyms for COLIPA C146 in the COLIPA summary.

Ref. : 11

2.9. Carcinogenicity

No data

2.10. Special investigations

A small number of scientific publications were included in the dossier, but not described in the COLIPA summary. They are reviewed briefly here :

Substituted 1,4-naphthoquinones vs. the ascitic sarcoma 180 of mice

A series of 1,4-naphthoquinones were tested for activity against the ascitic form of sarcoma 180 tumour in mice. 2-Hydroxy-1,4-naphthoquinone was considered to have poor antitumour activity.

Ref. : 14

Some aspects of activity profile of sodium lawsonate in mice and rats

This paper does not include information on 2-hydroxy-1,4-naphthoquinone

Ref. : 15

Mutagenicity of natural naphthoquinones and benzoquinones in the Salmonella/microsome test

2-Hydroxy-1,4-naphthoquinone was mutagenic to *S typhimurium* strain TA2637 with metabolic activation (not tested in the absence of S9), but not to TA98 or TA100. TA2637 is not used in routine mutagenicity testing, but the result does not modify the conclusions with respect to the genotoxicity of 2-hydroxy-1,4-naphthoquinone.

Ref. : 16

Non-mutagenicity of the hair dye, henna, in the Ames test

2-Hydroxy-1,4-naphthoquinone was mutagenic to *S typhimurium* strain TA98 at 500 to 1000 µg/plate in the absence of metabolic activation. Henna (which contains 2-hydroxy-1,4-naphthoquinone at a concentration of about 1% in the dried leaves) was not mutagenic up to 1000 µg/plate. This result does not modify the conclusions with respect to the genotoxicity of 2-hydroxy-1,4-naphthoquinone.

Ref. : 17

These publications do not supply relevant supplementary information for the safety evaluation of COLIPA C146.

2.11. Safety evaluation

CALCULATION OF THE MARGIN OF SAFETY

Lawsone
(Non-oxidising)

NOT APPLICABLE

Based on a usage volume of 35 ml, containing at maximum xx %

Maximum amount of ingredient applied	I (mg)	=	
Typical body weight of human		=	60 kg
Maximum absorption through the skin	A (%)	=	
Dermal absorption per treatment	I x A	=	
Systemic exposure dose (SED)	I x A / 60 kg	=	
No observed adverse effect level (mg/kg) (rat, 13-week study)	NOAEL	=	
Margin of Safety	NOAEL / SED	=	

2.12. Conclusions

This substance has been tested, generally to appropriate guidelines and GLP. It is moderately toxic on acute ingestion, with mortalities occurring at 300 mg/kg and above.
Two 13-week studies have shown clear signs of toxicity to the haemopoietic system, kidney, forestomach and liver. Effects were seen as low as 7 mg/kg bw/day and the NOAEL was 2 mg/kg bw/day.

It was shown to be mildly irritant to the rabbit eye but not to cause appreciable skin irritation. Studies on sensitisation are equivocal.

Studies on mutagenicity/genotoxicity are sub-optimal in design and presentation of the results. This substance is mutagenic and clastogenic *in vitro* and *in vivo* in some experiments. It induced mutations and chromosome aberrations in mammalian cells *in vitro.* There were positive results in bone marrow micronucleus assays. At present, the available negative studies do not override the positive results.
According to Commission Directive 67/548/EEC relating to the classification, packaging and labelling of dangerous substances, lawsone seems to fulfil the criteria to be classified as a category 3 mutagen.

In vitro percutaneous absorption of a 2.0% solution of lawsone in a hair dye formulation amounted to 2.6 ± 1.8 μg/cm^2/24h.

Overall, it is concluded that, based on the present available information, this substance is not suitable for use as a non-oxidising colouring agent for hair dyeing and, by extension, is not suitable for any other cosmetic use(s).

2.13. Opinion

Lawsone is toxic, showing toxicity to the kidney, forestomach and haemopoietic system following repeated oral dosing at doses in the region of 7-20 mg/kg bw/day. The NOAEL is 2 mg/kg bw/day.

Lawsone is mutagenic and clastogenic *in vitro* and *in vivo* in some experiments.

The SCCNFP is of the opinion that, based on the present available information, lawsone is not suitable for use as a non-oxidising colouring agent for hair dyeing and, by extension, is not suitable for any other cosmetic use(s).

2.14. References

Submissions II and III included as well several published scientific papers on general issues of genetic toxicology, not related with the evaluation of genotoxic potential of Lawsone, or copies of protocols or recommendations. No attention has been given by this report to these papers.

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