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

OPINION

CONCERNING

LAWSONE

COLIPA n° C146

adopted by the SCCNFP on 16 February 2004
by means of the written procedure

Foreword

The original request to the SCCNFP was in 1999. The SCCNFP was 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?

The opinion adopted by the SCCNFP during the 16th plenary meeting of 13 March 2001 was based on this first submission from May 1996. In this opinion, Lawsone was given Classification 2A : the available data support the conclusion that the substance constitutes a health hazard.

This was based on three points :

1. The toxicity of lawsone; effects to the kidney, forestomach and haemopoietic system following repeat oral dosing at doses in the region of 7-20 mg/kg bw/day. The NOAEL is 2 mg/kg bw/day.
2. Percutaneous penetration of 0.374% is assumed from the available information, but the study conditions were not adequate and this value could be a considerable underestimation.
3. Lawsone is clearly mutagenic and clastogenic *in vitro* and *in vivo* (genotoxic substance, category 3 according to Directive 67/548/EEC relating to the classification, packaging and labelling of dangerous substances)

In the light of this opinion, the SCCNFP was requested to evaluate 5 further submissions. Submission II, August 2001; Submission III, February 2002; Submission IV, July 2002. Submission V, July 2003 was new data on the *in vivo* bone marrow micronucleus test in mice treated orally with Lawsone C 146, as well as a study on pharmacokinetics on rats treated with Lawsone C 146 (2). On 26 September, COLIPA provided a draft report on the potential of Lawsone C 146 to induce DNA damage. The final report of this study was submitted on 22 October 2003.

The SCCNFP was requested to inform the Commission whether these new results justified a modification of the opinions on Lawsone adopted during the 16th plenary meeting of 13 March 2001. Opinions were adopted at the 19th plenary meeting of 27 February 2002 and the 21st plenary meeting of 17 September 2002. It was reiterated that the SCCNFP considered, 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).

At the same time the Commission requested a stringent review of the mutagenicity and carcinogenicity data of hair dyes in the light of the papers from Gago-Dominguez.
(1. Gago-Dominguez,M., Castelao,J.E., Yuan,J.-M., Yu,M.C. and Ross,R.K. (2001) Use of permanent hair dyes and bladder cancer risk. *Int. J. Cancer*, **91**, 575–579; Gago-Dominguez,M., Chan,K.K., Ross,R.K. and Yu,M.C. (2001) Permanent hair dyes and bladder cancer risk. *Int. J. Cancer*, **94**, 905–906.)

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 amalgamate all the opinions in light of the new data and 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

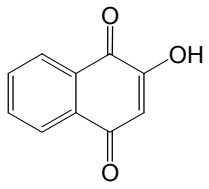
2.1.3. Trade names and abbreviations

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

2.1.4. CAS no.

CAS no : 83-72-7
EINECS no :

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 in batch 8160:FE. (methanol, ethanol, isopropanol, n-propanol, acetone, ethylacetate, cyclohexane, methyl ethyl ketone and monochlorobenzene < 100 ppm)

2.1.8. Physical properties

Appearance	:	Yellow to mustard coloured powder
Melting point	:	194.5°C
Boiling point	:	/
Density	:	/
Rel. vap. dens.	:	/
Vapour Pressure	:	/
Log P_{ow}	:	/

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.

No other existing uses are mentioned.

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%) or 97%
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 iridial 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 by the study authors.
Based upon the observed reactions, the substance should be regarded as irritant to the rabbit eye. The evaluation of erythema was obscured by orange colouration caused by the test substance during the first hour.

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. The histopathological cutaneous changes in the treated animals were equivocal since they did not correspond with those described in the literature for hypersensitisation and were comparable to the controls. However, the slides were peer-reviewed 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.
Evaluation of sensitisation potential based on this evidence was not possible since the irritant effect properties masked the sensitisation effect.

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.

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

Guideline	:	OECD draft guidance document and SCCNFP
Tissue	:	Human dermatomed skin
Method	:	Franz diffusion cell (static)
Test substance	:	2-Hydroxy-1,4-naphthoquinone, 2.04% ± 0.13% in vehicle
Batch no	:	21 (purity: 99.5%)
Vehicle	:	Epicea (60%) and water (40%)
Dose levels	:	20mg formulation
Replicate cells	:	8 cells
GLP	:	in compliance

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/cm^2 of formulation were applied to the skin specimen, whereas 2 mg/cm^2 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 amount of Lawsone is given as 2.6 ± 1.8 µg/cm^2. Consistent with the protocol provided, this is the absorption over 24h, so it can be interpreted as 2.6 ± 1.8 µg/cm^2/24h.

Ref. : 40

2.7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption, distribution and elimination *in vivo*

Species/strain	:	Male and female, Sprague Dawley rats: Crl CD(SD)IGS BR			
Age	:	7-8 weeks at start			
Groups	:	pharmacokinetics,	oral	group 1	12 rats/sex
			dermal	group 2	12 rats/sex
		excretion balance	oral	group 3	5 rats/sex
			dermal	group 4	5 rats/sex
Test substance	:	Radiolabelled [^{14}C]-2-hydroxy-1,4-naphthoquinone; 1.80 Bq/mmol, 48.67mCi/mmol; radiochemical purity 99%, diluted with non-radiolabelled 2-hydroxy-1,4-naphthoquinone (Lawsone); Titer (by potentiometry) 99.5g/100g.			
Oral exposure	:	Gavage; 2.2 MBq/kg in 10 ml 0.5% carboxymethylcellulose/kg bw (4.9 mg 2-hydroxy-1,4-naphthoquinone/kg bw)			
Dermal Dose	:	3.7 MBq/kg in 240 mg cream (Baum à l'épicéa)/kg bw (2% test substance in cream; equivalent to 4.9 mg 2-hydroxy-1,4-naphthoquinone/kg bw).			
Exposure time	:	30 min exposure followed by washing			
Exposure surface	:	25 (for 200 g rat) -30 (for 250 g rat) cm^2			
		2 mg cream/cm^2 (= 0.039 mg 2-hydroxy-1,4-naphthoquinone/cm^2)			
Pharmacokinetics	:	Up to 24 hours[1] post administration			
Excretion balance	:	Up to 168 hours post administration			
GLP	:	In compliance.			

[1] The 48 h (final) time points were excluded from pharmacokinetic modelling because of 'aberrantly high' plasma radioactivity levels for the cutaneously treated animals.

Results Pharmacokinetics (group 1 and 2)

* Following oral dosing, the plasma activity levels increased to a C_{max} at 0.5 h (ca. 15.000 and ca. 18500 ng-eq/g males/females respectively). After 168 h, levels were still quantifiable (95 and 134 ng-eq/g males/females respectively).

  Following topical application for 30 minutes, a much lower C_{max} was reached at 1-4 h. (ca. 50 and ca. 140 ng-eq/g males/females respectively). At 168 h. plasma levels were no longer quantifiable.

  The calculated plasma AUC $_{0-\infty}$ levels following oral exposure were 81528 and 126469 ng-eq/g*h males/females respectively.
  After cutaneous applications these levels were 1098 and 1312 ng-eq/g*h males/femalesrespectively.

* Systemic exposure after a topical dose for 30 minutes was estimated to be in the range of 0.74-1.35% of the systemic exposure after a similar oral dose based on AUC values.

Results Excretion balance (group 3 and 4)

* After oral dosing, the recovery of radioactivity was high (93-95%). Most activity was excreted with the urine (at least 66% but probably much more (88-90%). There was uncertainty as there was cross-contamination with cage wash). Approximately 5% was excreted with the faeces.

* Following oral dosing, up to >90% radioactivity was eliminated in the excreta within 24 h. (Whole carcass radioactivity was not determined after oral dosing).

* After topical application, the radioactivity in excreta was low (ca. 4% with urine and ca. 3% with faeces). The total recovery of radioactivity (skin swaps + cage wash + excreta + skin + carcass) was low (ca. 63%). The low total recovery was ascribed to evaporation of radiolabelled test mixture before washing the test site.

* Following topical dosing, the radioactivity in the excreta was eliminated, in a decreasing rate over time, over the complete 168-h collection period. The radioactivity recovered at necropsy in the skin (dermis and epidermis) and carcass was :

	radioactivity in skin at 8 h	radioactivity in skin at 168 h	radioactivity in carcass at 168 h
Males	10.4%	5.0 %	1.9%
Females	6.5%	2.8 %	0.9%

The radioactivity levels in skin strips were low and showed a decrease over time and with increasing depth of the stratum corneum.

Comments
In the pharmacokinetic study the internal dose following oral and dermal administration was compared. Based on AUC_{0-t} and $AUC_{0-\infty}$ values, the systemic exposure after a topical dose was much lower (in the range of 0.74-1.35%) than after an oral dose.

However, after 168 h, 2.8 - 5% of the topically applied radioactivity was still present in the skin while 0.9-1.9% was present in the carcass (the carcass was not analysed after oral dosing). The activity released from skin and/or carcass in time is theoretically represented in the $AUC_{0-\infty}$ after oral or dermal dosing. All 48 h (final) time points were excluded from pharmacokinetic modelling because of 'aberrantly high' plasma radioactivity levels for the cutaneously treated animals. The insufficient adequate time points would seem to result in low estimates of the plasma $AUC_{0-\infty}$ levels for cutaneously treated animals.

The validity of the comparison of the systemic exposure on basis of the AUC levels after oral and dermal dosing may be, therefore, doubtful (the continuing release from the skin depot may have been underestimated).

The recovery in the dermal excretion balance study was poor. The study authors suggested that ca. 35% lawsone may have evaporated from the applied cream over a 30 minute period. This is a very strange hypothesis that would have been feasible to have tested.

Nevertheless, the results of this study show that the systemic exposure following dermal application under "worst case in-use conditions" {direct application on rat skin (10% surface

area) of 2% lawsone in a cream (2 mg cream/cm^2)} is considerably lower than systemic exposure resulting from oral administration.

Despite the poor recovery in the excretion balance study, it may be concluded from this study that at least 17.5% (males) or 11.8% (females) of the dose topically applied had penetrated the skin, still present in the skin depot or available systemically after 168 h. This was equivalent to at least 6.8 µg/cm^2 (males) or 4.6 µg/cm^2 (females).

Ref. : 44, 48

2.8. Mutagenicity/Genotoxicity

2.8.1 Mutagenicity/Genotoxicity *in vitro*

LAWSONE: Summary of *in vitro* tests submitted

SUBMISSION	TEST	Submission ref	COLIPA	SCCNFP Opinion	Literature
I (October 1995)	SALMONELLA (CIT: April 1994)	6	NEGATIVE	NEGATIVE (13.3.2001)	POSITIVE in strain TA1537 in the presence of 10% S9 mix from Rat liver. NTP (Env. Mut, 8, (7)1-119, 1986)
	MOUSE LYMPHOMA (UK: January 1992	7	POSITIVE	POSITIVE (13.3.2001)	
	CHROM. ABERR. (CHO) (CIT: May 1994)	8	POSITIVE (+S9)	POSITIVE (13.3.2001)	
II (June 2001) requesting a Re-evaluation	SALMONELLA (Pub. 1979: HENNA)	17	NEGATIVE (Material identity not clear)		
	SALMONELLA (Pub. 1983: Natural NQ)	16	POSITIVE (Material identity not clear)		
	CH.V79 HPRT (CIT 1996)	19	NEGATIVE	INADEQUATE (before OECD Revision) (27.02.2002)	
III (02.2002) Additional information From the literature	No new experimental genotoxicity/mutagenicity data.. Negative data from the literature				NTP Salmonella assays, Lawsone may be positive. Lawsone may be genotoxic in vitro, although there are some doubts on the validity of some studies
IV (July 2002) Consultants' & Experts' statements	Statement By: Prof. D.Marzin (France) Prof. H.W. Marquardt, Germany Dr. D.Kirkland, UK Porf. G.Speit, Germany Dr. R.Glomot, France	45			
V (20 Sept. 2002) (22 Oct 2003)	Detection of oxidative DNA damage in cultured Chinese Hamster Ovary (CHO) cells using the Comet Assay.	49	NEGATIVE	POSITIVE Moderate induction of DNA repair NEGATIVE for DNA oxidised bases INADEQUATE as data gaps	

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

The test substance has been investigated for gene mutation in *S. typhimurium* and *E. 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

The test substance 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 metabolic activation)
GLP	:	OECD G.L.P. (1981)

The test substance 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 (N'-methyl-N'-nitro-N-nitrosoguanidine MNNG; benzo(a)pyrene BaP).

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., This study provides equivocal results in accordance with OECD guidelines. This should be clarified by further testing preferably using a modification of experimental conditions.
The study presented was performed during 1996. The 476 OECD Guideline was updated in 1997.

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	:	in compliance

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 h 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 was updated in 1997. The study presented was performed during 1994).

Ref. : 8

2.8.2. Mutagenicity/Genotoxicity *in vivo*

LAWSONE: Summary of *in vivo* tests submitted

SUBMISSION	TEST		COLIPA	SCCNFP Opinion (date)
I (October 1995)	Micronucleus study 1: MICE NMRI BR (AUSTRIA: 1989)	9.1	POSITIVE 250 mg/kg bw, 72h 72h harvest	POSITIVE (13.3.2001)
	Micronucleus study 2: MICE NMRI BR (AUSTRIA: 1990)	9.2	POSITIVE 110 and 250 mg/kg bw, 72h harvest	POSITIVE (13.3.2001)
	Micronucleus study 3: MICE SWISS 0F1/ICO: 0Ff1BR (France: 1994)	9.3	NEGATIVE 30, 100, 300 mg/kg bw 24, 48h harvest	INADEQUATE No 72h harvest (13.3.2001)
	Chromosome aberration: Chinese Hamster (FREIBURG 1992)	10	NEGATIVE 200 mg/kg bw at 6, 24, 48h	INADEQUATE No 72h harvest (13.3.2001)
II (June 2001) requesting a Re-evaluation	Unscheduled DNA synthesis: RATS (Germany 1993)	11	NEGATIVE	NEGATIVE (13.3.2001)
	Micronucleus study 4: MICE SWISS (France 2001) s	31	NEGATIVE 250 mg/kg bw, 72h harvest	INADEQUATE (no positive control) (27.02.2002)
	HENNA ROT Micronucleus study	42	NEGATIVE 300 mg/kg bw, 24, 48, 72h harvest	NOT CONSIDERED (test substance Lawsonia inermis) (17.09.02)
	Chromosome aberration (bone marrow): MICE SWISS (France 2001)	38	NEGATIVE 250 mg/kg bw	NEGATIVE (17.09.02)
	Chromosome aberration (peripheral lymphocyte): RATS 28 days (UK 1992)	18	NEGATIVE 12.5, 25, 50, 100 mg/kg/day 3 days	INADEQUATE (Unusual protocol, no reference material) (27.02.2002)
III (02.2002) Additional literature data	No new *in vivo* data			
IV (July 2002) Consultants' & Experts' statements	Statements By: Prof. D.Marzin,France* Prof. H.W. Marquardt, Germany Dr. D.Kirkland, UK * Porf. G.Speit, Germany Dr. R.Glomot, France	45	The weak positive response in 2 micronucleus studies might be due to the haematotoxicity of Lawsone or DMSO * D. Kirkland, D Marzin:An Assessment of the genotoxicity of 2-hydroxy-1,4-naphthoquinone,the natural dye ingredient of Henna.: Mutation Research 537 (2003) 183-199 Discussion of all the data provided by the Industry:	No haematotoxicity was reported in the studies presented by COLIPA
V (20 Sept. 2002) (22 Oct 2003)	Micronucleus study 5: MICE NMRI BR (France 2003)	47	NEGATIVE	NEGATIVE

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%) Shipping label FC 20048
Stability	:	> than 1 year
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 micronucleated polychromatic erythrocytes (MPE) 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%) Shipping label FC 20048
Stability	:	> than 1 year
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 micronucleated 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 micronucleated polychromatic erythrocytes at either harvest time. The PE/NE ratio was significantly lower for all doses at the 24 hour harvest, by the 48 hour harvest only at 30 and 300 mg/kg. This indicates that the test substance had reached the bone marrow. The positive control agent gave the expected results.
The study authors concluded that the substance did not induce cytogenetic damage under the conditions of the assay.

Ref. : 9.3

Mouse bone marrow micronucleus test – fourth study

OECD guidelines	:	Not followed. This study performed according to Schimd (1975) and modified by Salamone et al. (1980). Completed July 2001. The OECD 474 Guideline, adopted May 1983, updated July 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
Purity	:	>98.5%HPLC, ACROS Organics, Belgium
Batch No.	:	A0147608
Positive control	:	/
Negative control	:	0.5% methylcellulose.
Sacrifice time	:	72 hours after treatment
GLP	:	OECD GLP, 1997

The animals were treated orally and killed after 72 hours. For each animal haematology 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 : The treated group, compared with the control group, showed a similar frequency of MPE and similar PE/NE ratio.
The study indicates that the chemical is non-toxic for the bone marrow cells of mice.

Haematology investigations : 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, COLIPA C146, 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

Mouse Bone Marrow Micronucleus Test - fifth study

OECD guidelines	:	following the method according to Schimd (1975), modified by Salamone et al. (1980) and the OECD Guideline 474 (1997). Study completed 13.07.2001
Species/strain	:	NMRI CRL/BR mice (Charles River Lab France, Germany) 6 weeks old
Group size	:	5M +5F/dose
Dose level	:	75, 150 and 300 mg/kg (single oral)
Test Substance	:	100, 2-hydroxy-1,4-naphthoquinone
Purity	:	(>98.5% HPLC) ACROS Organics, Belgium
Batch No.	:	21
Vehicles	:	0.5% methylcellulose (MC) aqueous suspension dimethyl sulphoxide (DMSO)
Positive control	:	Cyclophosphamide (CPA) 50 mg/kg in water
Harvest times	:	24, 48 and 72 h
GLP	:	OECD GLP, 1997

Experimental start date was April 15th 2003, but the test substance, 100 (Batch 21) was analysed on January 21st 2002. There is no information on stability.
The doses were selected from a preliminary range finding study with the test substance in either 0.5% aqueous suspension of methylcellulose (0.5% MC) or in DMSO. Mortalities occurred at 375 mg/kg bw in either MC or DMSO.
The SCCNFP assumes that the single 'oral' dose (treatment volume 5 ml/kg bw) was administered by gavage or intubation "since it is expected to ensure an absorption of the test item is at least equal to the cutaneous route".
The test substance was administered at three dose levels, 75, 150 and 300 mg/kg in DMSO and in MC; negative controls: DMSO and MC only; Positive control: Cyclophosphamide (CPA) 50 mg/kg.

Dose	Harvest
75 and 150 mg/kg	24h
300 mg/kg	24, 48, 72h
CPA 50 mg/kg	24h

Mortality was observed in mice given the highest dose 300 mg/kg within 2 h (MC: 2 females, DMSO: 4 males and 1 female). These were replaced by a supplementary group.
For each animal haematology parameters were evaluated and the number of the micronucleated polychromatic erythrocytes (MPE) was counted in 2000 polychromatic erythrocytes. The PE/NE ratio was established by scoring 1000 erythrocytes.
Plasma level of the test item was determined for all doses in both vehicles (MC/DMSO) 30 min, 1 h and 4 h after treatment.

Results
Ratio PE/NE: a slight decrease was noted ($p<0.05$) in the DMSO 300 mg/kg treated males at 24 hours. No differences in the frequencies of MN were noted between all treatments and the controls. Plasma levels depended on the dose, with a C_{max} during the first hour after the treatment. There were no significant haematologocial abnormalities except reduced haemaglobin levels were seen in females treated with 300 mg/kg in DMSO.

Ref. : 47

In vivo mammalian bone marrow cytogenetic test in mouse chromosomal analysis

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 250 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 with the vehicle control group either 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. : 38

28-day _in vivo_ cytogenetic assay

OECD guidelines	:	/
Literature guidelines	:	Unknown

Species/strain	:	Sprague-Dawley CD
Replicates	:	3 animals/sex/dose
Doses	:	vehicle control; 12.5, 25.0, 50, 100 mg/kg/day for 28 days
Genotoxic endpoint	:	chromosome aberrations in rat peripheral lymphocytes
Test Substance	:	2-hydroxy-1,4-naphthoquinone
Vehicle	:	arachis oil B.P.
Positive control	:	cyclophosphamide
Batch	:	Lot 600522-32
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 (CPA).

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 (100 mg/kg/day) is reported.
No cytogenetic effect could be observed in the cultures derived from treated animals (vehicle 4.3%; C146 2.8%; CPA 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	:	in compliance

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.

Ref. : 11

Comet Assay

Introduction
A protocol for performing this test does not exist even as a draft at OECD or EC, but tentative guidelines for *in vitro* and *in vivo* Genetic Toxicology Testing, using the Comet assay has been discussed by a group of experts and published (R.R.TICE et al 2000 : ref,6).

COMET ASSAY measures increased DNA migration induced by:

- DNA strand breaks
- Alkali-labile sites
- DNA excision repair sites

Increased DNA migration induced by:

- Cross Links
- DNA-Protein Interactions

The pH of DNA unwinding determine the type of DNA damage assessed by Comet assay:

pH 7-8	:	Double strand breaks; Cross-links
pH12.1	:	Single strand breaks (SSB); Double strand breaks (DSB); Cross-links; Incomplete excision repair
pH>13	:	Alkali labile sites; SSB;DSB; Cross-links; Incomplete excision repair.

Comet formation is due to primary DNA lesions. For the interpretation of test results one should elucidate, whether the primary DNA damage is converted into biologically relevant chromosome

or gene mutations. Reactive oxygen species also induce chromosome instability. It is possible to investigate if specific oxidative DNA damages are induced with the Comet assay, by applying specific enzymatic systems on the treated cells. It has been demonstrated that DNA double strand breaks are not induced by treating the CHO cells with hydrogen peroxide (7).

Results
This study, as declared by the Study Director (not signed) was a 'non-regulatory' screening study. No raw data or report audit has been performed by the study laboratories QA unit, and therefore no claim of GLP compliance is made for this study.
Two independent preliminary cytotoxicity studies with and without metabolic activation were made, using 18 doses between 13.09 and 1742 µg/ml. The treatment lasted for three hours. The nature of the metabolic system employed was not indicated; the highest 3-4 doses were found to induce no toxicity (979.9; 1306; 1742 µg/ml).
The alkaline Comet assay was performed with three doses (435.5; 871; 1742 µg/ml); the maximum corresponding to 0.01M; the treatment (3 hours) was performed in the presence and in the absence of a metabolic activation system (S-9); the positive controls were represented by 4NQO and CPA. The slides were treated at pH > 13.0. No data are presented for the results. However, it is stated that the positive controls exhibited extensive DNA migration, and that the dose of 1742 µg/ml of Lawsone "showed a moderate degree of DNA migration" in both metabolic conditions.
This dose was selected for an enzyme treatment to evaluate the types of DNA damages. A second experiment, in which slides were exposed to the action of the enzyme formamidopyrimidine glycosylase (FPG), to identify the 8-OHdG DNA sites breaks. Only one slide showed a value proximal to a positive effect compared with hydrogen peroxide (200 µM) the positive control.

The substance was demonstrated to be clastogenic in the presence of metabolic conditions at the dose of 0.03 M on CHO cells. It may be concluded, on the base of this study, that 2-hydroxy-1,4-naphthoquinone has produced a positive "moderate" effect in the Comet assay, but it has not induced oxidative DNA damages (SSB).

Ref. : 49

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.

2.12. 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.
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.

In the radio-labelled pharmacokinetic study in rats, a single oral dose of 4.9 mg/kg/bw in methylcellulose, showed quick uptake into the plasma within 30 min (C_{max} of 14979/18545 ng-eq/g). There was a steady decrease down to 95.3/134 ng-eq/g by 168 h. The blood profile was similar. Most radioactivity (93-95%) was quickly eliminated in the excreta within 24 h.

A single topical application at 4.9mg/kg/bw for 30 min gave mean plasma levels reached of C_{max} of 141 ng-eq/g in females) at 1 h decreasing up to 24h. In males, plasma C_{max} 51.8 occurred at 4 h and stayed at a plateau 22.3 ng-eq/g. They were only quantified up to 48 h.

Radioactivity in excreta was low (ca. 4% with urine and ca. 3% with faeces). The total recovery

of radioactivity was also low (ca. 63%). This low total recovery was explained as "evaporation" of radiolabelled test mixture before washing the test site.

Despite the poor recovery in the excretion balance study, it may be concluded from this study that at least 17.5% (males) or 11.8% (females) of the dose topically applied had penetrated the skin, was still present in the skin depot or available systemically after 168 h. This was equivalent to at least 6.8 μg/cm^2 (males) or 4.6 μg/cm^2 (females).

Lawsone was found to induce gene mutations in bacterial cells (Salmonella TA1537 in the presence of metabolic activation, and in mammalian cells (mouse lymphoma) in the presence and in the absence of metabolic activation; it was found to induce chromosome aberrations in mammalian cells (CHO) in the presence of metabolic activation; it was found to induce a "moderate" positive effect in the Comet assay (CHO).

The chemical was found not to induce gene mutations and DNA oxidised bases in CHO mammalian cells.

5 *in vivo* tests for the induction of Micronucleus in the bone marrow cells of mice treated orally, with the following results:

2 studies on NMRI mice (aged 9 weeks) gave positive results 72 hours after treatment (confirmed in 2 experiments; dose-dependent activity).
1 study on NMRI CRL/BR strain (6 weeks of age) produced negative results at 24, 48, 72 hours after treatment

2 inadequate studies on Swiss 0F1/ICO produced negative results. Inadequate as :

1. no 72 h harvest evaluated; no demonstration that the compound has reached the target cells;
2. as no positive control was included.

Other *in vivo* tests included:

* the induction of UDS in hepatocytes of rats treated for 2 and 16 hours and was negative.
* the bone marrow cells of Chinese hamster treated orally for 6, 24, 48 hours with 200 mg/kg for the induction of chromosome aberrations. This study was considered inadequate because no 72 hours treatment was evaluated.
* the induction of chromosome aberrations in peripheral lymphocytes of rats treated for 28 days. This study was considered inadequate because no reference chemicals were included in the report.

Reappraisal of data

In the two 90-day rat studies submitted, there were no treatment related mortalities at doses up to 50 mg/kg/day.

During these experiments, treatment related responses were consistent though the amplification of the response seemed to be dependent on the vehicle. This was seen clearly in the terminal body weights. Comparable bodyweight gain and food consumption to controls were seen if lawsone was dosed as a suspension in methylcellulose, but with lawsone in arachis oil the

bodyweights were 98%, 93% and 90% of control at doses of 8, 20 and 50 mg/kg bw/day, respectively.

Hyper-salivation was reported in the dose range 50 and 20 mg/kg/day mainly in males from week 2 onwards.

Dose-related coloration of the urine with staining of the fur and tail was noted from as early as day 2 at the highest dose in arachis oil, 50 mg/kg/day and as delayed as week 10 in animals dosed at 20 mg/kg bw/day suspended in methyl cellulose. The bladder epithelium was found to be stained at necropsy. The increased water intake at the highest dose was possibly indicative of the development of renal dysfunction.

At necropsy, findings were consistent at all doses between the 2 studies despite the different vehicles, methylcellulose and arachis oil. Significant dose-related increases in relative kidney weights (male 112% -124% and females 115% - 129%). Relative liver weight in the females ranged from 108% - 131%, respectively. Relative spleen weights were significantly elevated in the mid and high dose animals (males: 123% - 161%; females: 128% - 188%, respectively. These weight increases mirrored macroscopic and microscopic changes.

Macroscopically there was mottling of the kidney. Microscopically there was evidence of renal tubular basophilia/dilatation/degeneration in both sexes at high dose. Haemosiderin was identified as the tubular pigment with increasing doses from 20 mg/kg bw/day. This was considered to be a sign of haemolytic anaemia and its appearance noteworthy as it is not usually observed spontaneously in the kidney.

Extramedullary haemopoiesis and haemosiderin accumulation 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 with increasing doses from 20 mg/kg bw/day.

In the stomach, there were dose related changes in both sexes, such as focal or multifocal ulceration of the mucosa, interstitial oedema with subepithelial inflammatory cell infiltration, acanthosis, hyperkeratosis.

There was a significant dose-related decrease in erythrocyte count in females with increasing doses from 7 mg/kg bw/day. This decrease was also seen in males at the top dose.

There were signs of haemolytic anaemia in females dosed at 50 mg/kg bw/day. These were decreased haemoglobin, erythrocyte counts and increased mean corpuscular volume and a significant, but not dose-related decrease in clotting time.

Dose-related increases in bilirubin also reinforced the observed haemolytic effects and the breakdown of haemoglobin.

Dose-related decreases in blood urea, creatinine were significant at 20 and 50 mg/kg bw/day in females, and albumin/globulin ratio was significantly decreased from 7 mg/kg bw/day. Creatinine was also significantly reduced in the males at the high dose.

A dose-related decrease in blood glucose and increase in triglycerides were also apparent in the males.

Munday (Ref. : SCCNFP 1) found 2-hydroxy-1,4-naphthoquinone to be both nephrotoxic and also to cause oxidative haemolysis. The nephrotoxicity was seen as renal enlargement and tubular necrosis, largely confined to the distal segment of the proximal convoluted tubules. Oxidative haemolysis is characterized by the presence of Heinz bodies in erythrocytes. Heinz bodies were not commented on the studies submitted. This type of haemolysis is thought to be glutathione dependent.
A single oral dose, that is rapidly excreted, would seem unlikely to induce the nephrotoxicity of lawsone within the timeframe of either the pharmacokinetic or micronucleus studies. There was no mention of discoloured urine in the micronucleus studies even at higher dose, 300 mg/kg bw in either methylcellulose or DMSO.
The haematocrits were all in the normal range of 31 to 52%. The study authors suggested that the second sample showed a decreasing tendency, indicative of slight anemia. This is cannot be substantiated as there were no readings at T_0.

The triggering of increased erythropoesis in the timeframe of either the pharmakinetic study (168h) or the micronuleus study (72 h) to the extent of producing micro-nucleated polychromatic erythrocytes would also be questionable.

The SCCNFP opinions are based on the view that the OECD guidelines should be considered together with biological relevancy. Therefore the *in vivo* micronucleus studies considered positive at 72 h by both COLIPA and SCCNFP (submission 1, 1989 and 1990) are still considered positive and biologically relevant by the SCCNFP, though discounted by COLIPA in latter submissions. These first studies and the 2003 *in vivo* mouse micronucleus were in the same strain of mice. The negative results observed in the 2003 study could be the result of the different sources of NMRI BRL/BR mouse strain used. Strain-sensitivity differences in mice are well documented in the scientific literature (SCCNFP 2, 3 and 4).
The purity of the chemical was stated to be between 98 –98.5%. In the 2003 experiment, the analysis of the chemical was 14 months prior to the experiment. There was no stability data in the recent experiment, but stability was given as over 1 year in the earlier studies. The batch numbers were not given for the first two tests, but was given for the 2003 test.

The OECD Guideline 474 (adopted: 26 May 1983) states that “animals are treated with the test substance once at the highest dose. Using the highest dose, samples of bone marrow are taken not extending beyond 72 hours”. The 1997 revision of the Guideline did not alter this but was to include micronucleus tests on mice peripheral erythrocytes.
The relevance of positive effect seen at 72h has been disputed. However it is known that chemicals have different latency periods for micronuclei induction and duration times vary. For example, MMC has a short latency period of 1.4 hours and a duration time for this effect of 47.3 hours. In 6-mercaptopurine (6-MP), micronuclei induction is seen at 48- and 72-h harvest times but not at 24 h and it has a duration time of 80.7 hours. This latency period is 10 hours longer than previously reported for other chemicals and the duration time is extended for 28 hours.

The UDS test in the livers of rats treated *in vivo* was negative. The other *in vivo* studies evaluated were inadequate due to a series of protocol deficiencies.

In all *in vivo* studies no haematotoxicity was mentioned. In the 2002 micronucleus study, there was a slight effect at the highest dose in one vehicle. It would seem unlikely that increased erythropoiesis could be triggered so rapidly.

Some of the other studies on mutagenicity/genotoxicity were sub-optimal in design and presentation of the results. This harmonised overall evaluation leads us to conclude that the weight of evidence of mutagenic potential of Lawsone should not be underestimated and that there is no robust proof that this molecule is devoid of genotoxicity/mutagenicity potential both *in vitro* and *in vivo.*

2.13. Opinion

After an overall re-evaluation of Lawsone, the SCCNFP is of the opinion that:

Lawsone shows toxicity to the kidney, stomach and haemopoietic system following repeated oral dosing.

The SCCNFP is aware that some of the genotoxicity/mutagenicity data is equivocal. However, on balance, the SCCNFP considers that Lawsone has genotoxicity/mutagenicity potential *in vitro* and *in vivo* and that therefore no safe threshold for Lawsone can be established.

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