EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

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

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

Lawsonia inermis (Henna)

COLIPA N° C169

Adopted by the SCCP
during the 6th plenary meeting of 13 December 2005

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1. BACKGROUND

Submission I for Henna was submitted in October 1999.
The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its 21st plenary meeting of 17 September 2002, an opinion (SCCNFP/0505/01, final) which stated that:

"S*ubmission I on Lawsonia inermis was inadequate. Before any further consideration, a full and adequate dossier would be required, including specifications of the substance tested and marketed, and adequate in vivo genotoxicity data on natural henna containing the maximum amount of 2-Hydroxy-1,4-naphthoquinone (Lawsone)".*

Submission II provided data on the specification of *Lawsonia inermis,* but it did not include the data requested on *in vivo* genotoxicity.

Submission III was submitted in July 2005 and provided data on the conditions of use of *Lawsonia inermis.* Submission III presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

Lawsone, a natural component in the hair dye preparations of *Lawsonia inermis*, has been examined by SCCNFP which in its latest opinion on Lawsone (SCCNFP/0798/04), adopted on 16 February 2004, stated that ' *Lawsone has genotoxicity/mutagenicity potential in vitro and in vivo and that therefore no safe threshold for Lawsone can be established*.'

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider Lawsonia inermis safe for use as a hair dye taken into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of Lawsonia inermis in any hair dye formulations?*

3. OPINION

This opinion concerns the evaluation of *Lawsonia inermis* (Henna) per se.

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

The plant Henna (*Lawsonia inermis*, family *Lythraceae*) is a shrub that is naturally grown or cultivated from north-east Africa to India. Marketed Henna represents a natural material derived from dried and powdered leaves of the plant.

3.1.1.1. Primary name and/or INCI name

Lawsonia inermis

3.1.1.2. Chemical names

Not applicable

3.1.1.3. Trade names and abbreviations

Henna, Henna powder, Lawsonia alba, Henna pulver, Henna Rot

3.1.1.4. CAS / EINECS number

CAS: 84988-66-9
EINECS: 284-854-1

3.1.1.5. Structural formula

Not applicable

3.1.1.6. Empirical formula

Not applicable

3.1.2. Physical form

Greenish-grey powder

3.1.3. Molecular weight

Not applicable

3.1.4. Purity, composition and substance codes

The natural constituents of *Lawsonia inermis* are essential oils, 1,4-naphthoquinone, tannins, gallic acid, flavonoids, lipids, sugars, triacontyl tridecanoate, mannitol, xanthones, coumarins (5-alkyloxy 7-hydroxycoumarin), 2-3% resins, 5-10% tannic ingredients and up to 2% Lawsone (2-hydroxy-1,4-naphthoquinone). A major portion of Lawsone is glycosidic bound, and that is cleaved by enzymatic hydrolysis of the glycosidic hennosids and autooxidation of aglucons.

Ref.: Dossier on *Lawsonia inermis*

Analysis of a Henna powder

	Content % (w/w)	
	Sample No 1271 from LOGCOS	**Batch No. 830.72**
Loss on drying	4.5	4.3
Total Ash	14.6	Max 15%
Lawsone	1.28(UV-spectrometry)/1.48(HPLC)	1.17(UV-spectrometry)
Flavonoids Rutosid Hyperosid	/ 1.54 not detectable	/ no information 0.79
Water soluble extract	5.0	32.9%

Comment
As the water soluble extract changes significantly from batch to batch, clarification is needed.

Ref. (2, 21)

In submission I, batch 830.72 was not properly characterized, which caused problems with the interpretation of especially the mutagenicity studies. In the present submission dossier, the authors refer to various study reports (subchronic toxicity, prenatal developmental toxicity and SCE study) where the specification of this batch is reported. The Lawsone content of the 830.72 batch is 1.17%.

Ref.: 3 (subm I), analytical file (subm II)

3.1.5. Impurities / accompanying contaminants

Sample No. 1271 from LOGOCOS

Pesticides
Investigation of contamination of Sample No. 1271 for 30 commonly used pesticides revealed absence of these pesticides in the sample (detection limits 0.01 -3 ppm depending upon the compound)

Metals
Lead 1.04 ppm, Cadmium 0.11 ppm, Mercury 0.03 ppm, Arsenic 0.47 ppm, Copper 7.87 ppm, Chromium 9.4 ppm and Nickel 8.06 ppm, Iron 4915 ppm

Ref.: 21 (subm. II)

Batch No. 830.72
Other accompanying substances: <2%

Ref. 2

3.1.6.	Solubility

Lawsonia inermis is partially soluble in water with varying degree of solubility, depending upon the composition of the sample.

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow} : /

3.1.8.	Additional physical and chemical specifications

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

3.2. Function and uses

Lawsonia inermis (Henna) is used as a hair dye based on the staining properties of one of its constituents, e.g. Lawsone.
Modified Henna products, such as Black Henna are also available to consumers. The content of Lawsone among various modified Henna products may vary significantly, but these products contain some other substances for modifying the intensity of the colour provided by Henna alone.

According to the information provided, a representative hair dye formulation will be prepared by mixing 100 g *Lawsonia inermis* as dried plant powder with 300 ml of boiling water. After cooling the mixture (mush) the pulp will be applied on the hair for a period of 15 min to 2h. Thereafter, the mush is rinsed off with water and the hair will be washed with a mild shampoo to eliminate any residues.

Ref.: 17 (subm. II)

Although *Lawsonia inermis (Henna)* is not listed in Annex IV of Directive 76/768/EEC on cosmetic products, aqueous pastes of Henna are used for skin decoration.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

An acute oral toxicity study of Henna Rot was performed in the Sprague-Dawley Rat according to the OECD Guideline N°401 (1981).
The calculated oral median lethal dose was > 2000 mg/kg bw.

Ref.: 1 (subm. I)

3.3.1.2. Acute dermal toxicity

An acute dermal toxicity study of Henna Rot was conducted in the Wistar Rat according to OECD Guideline 402 (1987).
Median lethal dose for Henna Rot was > 2000 mg/kg bw.

Ref.: 2 (subm. I)

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

No separate skin irritation study was performed in experimental animals.

Lawsonia inermis showed no irritative potential for the skin after a single occlusive application for 24 hours, when tested for acute dermal toxicity (described above) under the experimental conditions.

Ref.: 22 (subm. II)

3.3.2.2. Mucous membrane irritation

Guideline:	OECD guideline 405 (1987)
Species/strain:	Rabbit/New Zealand White
Group size:	1 male and 2 females
Test substance:	Henna rot
Batch:	830.72
Concentration:	green powder ‘as is’
Route:	instillation in the conjunctival sac of the right eye
Observation period:	7 days
GLP:	Yes

0.1 ml of the unchanged test substance (approximately 58 mg) was instilled into the conjunctival sac of the right eye of each of 3 New Zealand White rabbits (1 male, 2 females). The test substance was not washed out. Observation period was 7 days and the readings were performed

at 1, 24, 48, 72 hours and 7 days. The untreated eye served as control. The findings were scored according to a modified scoring system comparable to the scheme of Draize.

Results
No findings of the cornea occurred. Transient inflammation of the iris and moderate conjunctival irritation were observed up to a maximum of 48 and 72 hours, respectively. When scored, according to the modified scoring systems, the mean values are 17.0; 15.7; 10.0; 2.7 and 0.0 after 1, 24, 48 and 72 hours and 7 days, respectively. The iris and conjunctival findings subsided completely after 7 days.

Conclusion
Lawsonia inermis was slightly and transiently irritating to the eyes of 3 New Zealand White rabbits.

Ref.: 28 (subm. II)

3.3.3. Skin sensitisation

Buehler Test

Guideline:	OECD 406(1981), Commission Directive 84/449/EEC
Species/strain:	Guinea pig/Dunkin Hartley
Group size:	Main study: 10 female animals in the control, 20 female animals in the test groups
Test substance:	Henna rot
Batch:	830.72
Route:	Occlusive epicutaneous induction (50%) and challenge (50%)
Carrier:	Petrolatum jelly B.P.
GLP:	Yes

The sensitizing property of the test substance was evaluated in a Buehler delayed contact hypersensitivity study using female albino Hartley guinea pigs. One group of 20 animals received induction exposures of 0.5 ml at a concentration of 50% in petrolatum jelly B.P. on the left flank on absorbent lint (approximately 15 mm x 35 mm) after removal of hair. The occlusive dressing was kept in place for 6 hours and the induction was repeated on the same site on days 7 and 14 for a total of three 6 h exposures. The skin was carefully examined approximately 24 h after each induction on days 1, 8 and 15.
Challenge was performed on day 28 by application of 0.5 ml test material (50% in petrolatum jelly B.P.) on an area of about 15 mm x 30 mm on the right left flank clipped free of hair held under occlusion for 6 h. Approximately 24 and 48 h after removal of the occlusive dressing, the skin reaction was evaluated and scored using a four-point scale. The individual reactions to the tests substance preparation at the challenge sites were compared between control and test animals.

Results
Staining caused by the test substance preparation was observed after removal of the occlusive dressing. The study authors considered that this did not prevent evaluation of the treated skin in respect to erythema formation. No treatment-related reaction was noted on the treated skin in any animals (0/20).

Conclusion
The results suggest that *Lawsonia inermis* exhibited no potential to induce dermal sensitization in Guinea pigs in the Buehler test under the conditions used. However, skin staining may have compromised evaluation.

Ref.: 29 (subm. II)

Human data

Repeated insult path test (RIPT)

Guideline:	/
Species:	Human
Group size:	10 volunteers
Test substance:	Henna rot
Batch:	830.72
Route:	Epicutaneous application (no further information supplied)
GLP:	/

A repeated insult patch test (RIPT) according to a modified protocol was performed with the test substance on a panel of 10 volunteers. The tested concentration was 10% in petrolatum and the induction phase lasted for 3 weeks followed by a challenge after one week of no treatment.

Results
No skin findings were observed on the tested skin area of any of the volunteers at any time during the 3 weeks of induction phase and at challenge after a one week rest period.

Conclusion
There was no indication for any irritative or sensitizing potential under the conditions of the study in Human volunteers.

Comment
The study would currently no longer be considered ethical by the SCCP.

Ref.: 13 (subm. II)

Experience under specific conditions in humans

It is known from the literature that *Lawsonia inermis* is widely used both as a hair dye in and for skin paintings. Under such conditions of use, reports of contact allergies are rare. Two case reports from India describe allergic contact dermatitis from *Lawsonia inermis*.

Ref.: 19, 20 (subm. II)

A beautician with known allergy to house dust experienced rhinoconjunctivitis, asthma and a generalized urticaria after exposure to *Lawsonia inermis*. The symptoms increased in severity with continued exposure. Scratch tests with *Lawsonia inermis* powder were strongly positive. By thin-layer chromatography the red colour and 2-hydroxy-1,4-naphthoquinone were isolated from the extract. These materials gave negative scratch tests. The result showed that the allergen was neither the quinone nor the red colour but an undetermined agent. In a further case report, a

hairdresser had an immediate type hypersensitivity with urticaria, rhinitis, and bronchial asthma on exposure to *Lawsonia inermis*. Prick tests with *Lawsonia inermis* 1% *in aqua* and in ethanol showed positive reactions. Both patch tests and prick tests performed with 2-hydroxy-1,4-naphthoquinone, gave negative results.

Ref.: 10 (subm. I), 18 (subm. II)

3.3.4. Dermal / percutaneous absorption

Introductory remarks

Lawsonia inermis powder as a product of botanical origin is composed of various ingredients and cannot be examined *per se* for percutaneous absorption. Consequently, it is necessary to identify and select a representative lead ingredient. Since Lawsone is an important ingredient and can be analyzed easily, it has been selected as the lead ingredient. However, Lawsone is predominantly glycosidic bound and only a small amount is available in the plant powder. For practical and analytical reasons, Lawsone is therefore often added separately and mixed to the *Lawsonia inermis* powder, especially when the investigations were performed with radiolabelled material.

Percutaneous Absorption *in vitro*

Guidelines:	/
Test system:	Isolated pig skin
Method:	Permeation chambers (flow through system)
Test substance:	*Lawsonia inermis* powder (Henna containing 1% Lawsone (2-hydroxy-1,4-naphthoquinone))
Batch:	No data
Dose level:	25% *Lawsonia inermis* powder as aqueous preparation
Exposure period:	30 minutes
GLP:	No

The pig skin pieces were fixed into the permeation cells and 0.1 g/cm² of an aqueous pulp of 25% *Lawsonia inermis* powder (containing 1% of Lawsone) was exposed for 30 minutes. Then the residues were removed by a spatula and the skin was washed using water and detergent. Percutaneous penetration was determined after an incubation time of 72 hours. Following extraction, the amounts of Lawsone were analyzed by HPLC.

Results

When *Lawsonia inermis* powder was investigated under use conditions as a 25% aqueous pulp, it was shown that Lawsone penetrated through the pig skin *in vitro*. After exposure of 30 min and a follow-up period of 72 h, about 0.28% of the applied dose of Lawsone was found in the receptor fluid and 0.06% remained in the skin. Therefore, the respective absolute skin penetration rate was 703 ng/cm². The amount that remained in the skin was demonstrated as 160 ng/cm² but no differentiation was possible for the fraction that was adsorbed on the stratum corneum or absorbed by the deeper skin layers.

Ref.: 4 (subm. II)

An abstract was recently published on studies to mimic consumer use conditions of *Lawsonia inermis* containing products by investigating skin absorption of Lawsone from two hair colouring products and two shampoo products. [14C]-Lawsone was added separately to each commercial product and applied to excised, non-viable human skin (thickness of approx. 200 – 320 µm) mounted in flow-through diffusion cells perfused with a physiological buffer. The products remained on the skin for 5 min (shampoos) and 1 h (hair colour pastes). The examination of the henna hair paste products showed that 0.29 and 1.4% of the applied dose was absorbed into the receptor fluid in 24 h and 2.2% and 3.7% remained in the skin. For the henna shampoo products, 0.32 and 0.34% of the applied dose was absorbed into the receptor fluid at 24 h and 3.6% and 6.8% remained in the skin. For all products, most of the lawsone applied was washed from the surface of the skin (83 - 102%) at the end of the exposure period. Extended absorption studies for 72 h demonstrated that the majority of lawsone remained in the skin and the receptor fluid values did not increase significantly. Following administration of henna paste for example, the 72 h receptor fluid values were only 0.48 and 1.61% opposed to 0.29 and 1.4% after 24 h.

As only the abstract is presently available, the validity and reliability of these investigations cannot be assessed; important information on materials and methods, including analysis, are missing and the results are only provided as percentages and not as absolute values, which is a prerequisite for valid risk assessment.

Ref.: 15 (subm. II)

Percutaneous absorption *in vivo*

Guidelines:	/
Species/strain:	Rat/Sprague-Dawley (Him:OFA)
Group size:	5 animals per sex and group
Test substance:	23.5% *Lawsonia inermis* powder mixed with 75 % of deionized water and spiked with 1.5 % of [14C]-Lawsone (2-hydroxy-1,4- naphthoquinone, specific activity 11,375; 11,671 and 11,770 MBq/g) applied as aqueous pulp to mimic human use condition
Batch:	*Lawsonia inermis* (Henna rot): 830.72 Lawsone (unlabelled): 94028 (purity: 99.9%) [14C]-Lawsone: Synthesis by NEN, Boston USA (radiochemical purity: >96.5%)
Dose level:	i) 72 h sampling time: 0.509 g of aqueous pulp/animal corresponding to 7.64 mg *Lawsonia inermis* spiked with [14C]-Lawsone/animal or corresponding to 0.85 *Lawsonia inermis* spiked with [14C]-Lawsone/cm² ii) 24 h sampling time: 0.517 g of aqueous pulp/animal corresponding to 7.76 mg *Lawsonia inermis* spiked with [14C]-Lawsone/animal or corresponding to 0.86 *Lawsonia inermis* spiked with [14C]-Lawsone/cm²
GLP:	Yes

Prior to the administration of the aqueous pulp, the animals were anesthetized with 40 mg/kg bw thiopental i.p.. The preparation was spread with a spatula to an area of 3 x 3 cm to the dorsal, median thoracic to lumbar area. The dorsal skin of the animals was clipped one day before application of the test substance. Animals were held tightly during the contact period. The aqueous pulp was left for 40 min and then rinsed off. The treated areas were covered and the rats subsequently placed into the metabolism cages. One group (I) was sacrificed after 72 h and samples were drawn from rinsing water, treated skin, urine, feaces, organs (adrenals, blood,

brain, fat, femur, heart, kidneys, liver, lungs, muscle, ovaries, skin (untreated), spleen, testes, thyroids), carcass. The other group (II) was sacrificed after 24 h and radioactivity was determined in blood samples taken from the retrobulbar venous plexus (first sampling 45 min. p.a.).

Results
The majority of the applied radioactivity was removed from the skin by rinsing 40 min after application of the aqueous *Lawsonia inermis* pulp spiked with [14C]-Lawsone and a mean of 93.9% was detected. The mean percutaneous absorption of the test substance amounted to 0.20 % of the administered radioactivity after 72 hours. The application site contained a mean radioactivity level of 3.1% of the applied dose. The blood level of [14C] was highest at 45 min after application and declined with an initial half-life of approximately 2 h. The [14C]-labelled substance was excreted mainly via urine (86 % of the eliminated radioactivity) and to a lesser extent via faeces (14% of the eliminated radioactivity). Within the first 24 h, the mean excretion was fast, 91% of the eliminated [14C] substance. The mean radioactivity levels of blood and the 14 analyzed organs were all near or below the detection limit at 72 h after application. Relatively high concentrations were found in thyroids, kidneys and ovaries; lowest in muscle, heart and femur.

Conclusion
The cutaneous application of the aqueous *Lawsonia inermis* pulp spiked with [14C]-Lawsone to mimic human use conditions onto the skin of male and female rats rat skin led to a percutaneous absorption of 0.2% determined as radioactivity after 72 hours, corresponding to an absolute absorption of 1.70 µg /cm².

Comment
The exposure time in the study (40 minutes) did not comply with the exposure time used by consumers (15 minutes to 2 hours).

Ref.: 9 (subm. II)

3.3.5. Repeated dose toxicity

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

No data submitted

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

A 13-week oral toxicity study was conducted in Sprague-Dawley rats (4 groups of 10 rats per sex) with a 0.5 % aqueous methylcellulose solution of Henna Rot administered once daily by gavage according to the OECD Guideline N° 408. The treated animals received the test substance corresponding to daily dosage of 40, 200 and 1000 mg/kg body weight. Control animals received the vehicle alone under the same conditions. In addition, 10 males and 10 females were included in the control and high dose group for a 4-week recovery period.

No mortality was observed during the study. In the high dose group, 2/40 animals occasionally presented signs of poor clinical condition (loud breathing, piloerection) and 6/20 males presented ptyalism from week 9 or 11 onwards. Brown urine and/or tail were noted in almost all animals. All clinical signs were reversible after 4-weeks recovery period, except for brown-coloured tail.

In the high dose group, the hair and body extremities as well as the fore-stomach and the mucosa of the bladder were coloured orange related to the staining properties of the test substance. Mean food consumption and body weight gain of the treated males were in the range of controls; mean body weight gain of the females given 40 or 1000 mg/kg/day was slightly lower than that of control but this finding was not dose-dependent and was not considered by investigators to be treatment-related. Neither treatment-related ophthalmological abnormalities nor effects in clinical chemistry (blood biochemistry, urinalysis) were noted in any treated group.
Concerning haematological parameters, slightly lower erythrocyte count and haemoglobin were noted in the high dose group when compared to the control values, these differences were considered of no toxicological importance by the investigators. In the highest dose group, statistically significant higher mean kidney and spleen weights were noted.
All these findings were reversible after 4-weeks recovery period except for the kidney weight of the females, but it was considered to be of no toxicological importance by the investigators as no relevant microscopic findings were noted.

Concerning microscopic examinations, no findings of toxicological relevance were noted at the low dose level. In the 200 mg/kg/day group, minimal to slight hemosiderosis was noted in the spleen. In the high dose group, minimal to moderate accumulation of acidophilic globules in the cortical tubular epithelium of the kidneys were recorded and were considered to be treatment-related. In the 200 mg/kg/day and in the 1000 mg/kg/day groups, minimal to slight hemosiderosis and some extramedullary hemopoiesis were noted in the spleen. Except for the hemosiderosis in the spleen and the dyeing effects in the high dose group, all findings were reversible during the recovery period.

Based on these results, the NOAEL (No-Observed-Adverse-Effect-Level) of Henna Rot was established to be 40 mg/kg bw.

Ref.: 3 (subm. I)

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay (I)

Guideline:	OECD 471 (1983)
Test system:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA1538
Replicates:	triplicate plates, two independent assays
Test substance:	Henna rot
Batch:	830.72
Concentrations:	50 – 5000 µg/plate with and without metabolic activation
Solvent:	DMSO
GLP:	Yes

The test substance was tested for mutagenicity in the reverse mutation assay on bacteria both, with and without metabolic activation (S9 mix from the liver of Phenobarbital and ß-Naphthoflavone induced male Wistar rats) according to the plate incorporation method. The *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA1538 were exposed to the test substance (dissolved in DMSO) at concentrations ranging from 50 µg/plate to 5000 µg/plate (with and without S9 mix). All concentrations were filtered through a 0.45 µm filter.
Appropriate negative and positive controls were included.

Results
No bacteriotoxic effect was observed. Henna Rot did not induce revertants in the bacterial strains in the tested concentration range between 50 to 5000 µg/plate. The sensitivity and validity of the test system used was demonstrated by the significant induction of revertants by the positive controls.

Conclusion
In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frame shifts in the genome of the bacterial strains used in the presence and absence of S9-mix. Thus, Henna rot (batch 830.72) was shown to be non-mutagenic in this *Salmonella typhimurium* test.

Ref.: 5 (subm. II)

Bacterial gene mutation assay (II)

Guideline:	Not stated but corresponding to OECD 471 (1983)
Test system:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA1538 or streptomycin-resistant strains TA98strp, TA100strp, TA1535strp, TA1537strp, TA1538strp
Replicates:	triplicate plates, two independent assays
Test substance:	Henna rot
Batch:	830.72
Concentrations:	50 – 5000 µg/plate with and without metabolic activation
Solvents:	DMSO and water
GLP:	Yes

The test substance was tested for mutagenicity in the reverse mutation assay with bacteria both, with and without metabolic activation (S9 mix from the liver of Aroclor induced male Wistar rats) according to the plate incorporation method. The following experiments were performed with Henna Rot at concentrations ranging from 50 µg/plate to 5000 µg/plate (with and without S9 mix):

- suspended in water and tested in strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538
- suspended in water and tested in streptomycin-resistant strains TA 98 strp, TA 100 strp, TA1535 strp, TA 1537 strp and TA 1538 strp
- suspended in DMSO and tested in streptomycin-resistant strains TA 98 strp, TA 100 strp, TA 1535 strp, TA 1537 strp and TA 1538 strp

Appropriate negative and positive controls were included.

Results
No bacteriotoxic effect was observed but the test material showed bacterial contamination.
Therefore, an evaluation of the high concentrations was limited in the normally used tester strains. The application of streptomycin-resistant tester strains proved to be a useful way to test contaminated materials. These tester strains showed a comparable sensitivity and spontaneous reversion rate compared to the normally used strains. The advantage of the use of streptomycin-resistant strains is that the test material needs no sterilization. Henna Rot did not induce revertants in the bacterial strains in the tested concentration range between 50 to 5000 µg/plate.
The sensitivity and validity of the test system used was demonstrated by the significant induction of revertants by the positive controls.

Conclusion
Under the experimental conditions selected, Henna rot (batch 830.72) did not show a mutagenic potential in the presence and absence of S9-mix this *Salmonella typhimurium* gene mutation test. (Reference: 6)

The results of these two bacterial gene mutation assays are in line with data published in the literature. Stamberg et al. (1979) tested *Lawsonia inermis* (red henna) in bacterial gene mutation test (Ames test) and did not find a significant induction of mutations for concentrations up to 1000 µg/plate

Ref.: 34 (subm. II)

Mammalian cell gene mutation assay (I)
HPRT gene mutation test

Guideline:	OECD Guideline 476 (1984)
Test system:	V79 Chinese hamster cells
Replicates:	Two independent assays
Test substance:	Henna rot
Batch:	830.72
Concentrations:	1 – 200 mg/ml without metabolic activation
	10 – 1000 mg/ml (first test) with metabolic activation
	10 – 3000 mg/ml (repetition test) with metabolic activation
Solvent:	Water
GLP:	Yes

The potential mutagenic effect of Henna Rot in cultured mammalian cells was examined by the HPRT gene mutation test with V79 cells. In a preliminary cytotoxicity assay, the test substance was applied at concentrations between 100 - 1000 mg/ml with and without metabolic activation (rat liver S9-mix induced with Aroclor 1254).
Thereafter, two independent main assays were conducted using concentration ranges of 1 – 200 mg/ml without S9 mix and 10 – 1000 mg/ml or 10 – 3000 mg/ml with S9 mix. S9 mix was obtained from the liver of rats induced with Aroclor 1254. Test substance was suspended in water, which was also used as solvent control, whereas the positive control substances Ethyl Methanesulfonate (1 µl/ml, without S9 mix) and 7,12-Dimethylbenz(a)anthracene (10 µg/ml, with S9 mix) were diluted in the cell culture medium.

Results

Cytotoxicity was observed in the main test at a dose of 200 µg/ml without S9-mix and at of 2000 µg/ml with S9-mix. The test substance caused no increase in the number of mutant colonies when tested without S9-mix. With S9-mix, the first test showed no increase in the number of mutant colonies. However, in the duplicate test a non-dose-dependent increase to a maximum factor of 3.3 was observed at the lowest dose only, while higher dose levels showed value in the range of negative controls. Moreover, the observed increase did not reach the threshold defined for a positive test. The sensitivity and validity of the test system used was demonstrated by the significant induction mutants by the positive controls.

Conclusion
In conclusion, Henna Rot (batch 830.72) is not mutagenic in the HPRT gene mutation test with V79 cells under the test conditions used.

Ref.: 7 (subm. II)

Mammalian cell gene mutation assay (II)
$TK^{+/-}$ gene mutation assay (mouse lymphoma assay)

Guidelines:	OECD 476 (1998)
Test system:	Mouse lymphoma cell line L5178Y
Replicates:	Two independent experiments
Test Substance:	Henna (*Lawsonia inermis*, COLIPA n° C169)
Batch:	1271 (content of 2-Hydroxy-1,4-naphthoquinone 1.36%).
Concentrations:	Experiment I: 75 – 900 µg/ml with metabolic activation
	150 – 1200 µg/ml without metabolic activation
	Experiment II: 78.1 - 1250 µg/ml without metabolic activation
Solvent:	Deionized water
GLP:	Yes

The test substance was tested for the possible induction of mutations at the thymidine kinase locus using the cell line L5178Y. The assay was performed in two independent experiments, using two parallel cultures each. The first main experiment was performed with and without S9-mix and a treatment period of 4 h. S9 mix was obtained from the liver of male Wistar rats induced with Phenobarbital/ß-Naphthoflavone. The second experiment was performed in the absence of metabolic activation with a treatment period of 24 hours. Prior to the mutation assays a pretest for cytotoxicity was performed to determine the concentration ranges in the mutagenicity test.
The highest concentration used in the pre-test (5000 µg/ml) was chosen according to the guidelines. Test item concentrations between 39.1 and 5000 µg/ml were used to evaluate toxicity in the presence (4 h treatment) and absence (4 h and 24 h treatment) of metabolic activation. The test medium was checked for precipitation at the end of each treatment period (4 or 24 hours) before the test item was removed. The stability of the test substance in the solvent (deionized water) was analyzed in respect to Lawsone for a period of at least 4 hours.
Appropriate negative and positive controls were included.

Results
Precipitation of the test item was observed at all concentrations of the test item. Relevant toxic effects, indicated by a relative total growth or a relative cloning efficiency below 50 % occurred in the first experiment at 600 µg/ml and above without- and at 900 µg/ml and above with metabolic activation.

No biologically relevant and reproducible increase of the mutation frequency was observed in any of the experiments. An isolated positive response was seen after 4 hours treatment in the absence of S9-mix 1200 µg/ml. However, relative total growth was less than 3% under these conditions.
The positive controls showed a distinct increase in induced total mutant colonies.

Conclusion
Under the experimental conditions used the test substance did not induce mutations in the $TK^{+/-}$ gene mutation assay with the mouse lymphoma cell line L5178Y in the absence and presence of metabolic activation. Thus, Henna (*Lawsonia inermis*, COLIPA n° C169, batch 1271 containing 1.36% 2-Hydroxy-1,4-naphthoquinone) was shown to be non-mutagenic in this mouse lymphoma assay.

Ref.: 23, 25 (subm. II)

Mammalian cell gene mutation assay (III)
$TK^{+/-}$ gene mutation assay (mouse lymphoma assay)

In contrast to the above result, a former $TK^{+/-}$ mouse lymphoma gene mutation assay with *Lawsonia inermis* showed an increase in mutant frequencies with and without metabolic activation at high concentrations. This study was performed with Henna rot (batch 830.72) and it is concluded that this batch was mutagenic in this in vitro mammalian cell gene mutation test.

Ref.: 26 (subm. II)

Chromosome aberration test in cultured Human lymphocytes

Guideline:	OECD 473 (1981)
Test system:	Human lymphocytes
Replicates:	duplicate culture in a single experiment
Test substance:	Henna rot
Batch:	830.72
Concentrations:	78.13 – 625 µg/ml without metabolic activation
	156.25 – 1250 µg/ml with metabolic activation for 20 h harvest
	312.5 - 1250 µg/ml with metabolic activation for 30 h harvest
Solvent:	Culture medium (Eagle's Minimal Essential Medium (MEM))
GLP:	Yes

Henna Rot assessed for its potential to induce structural chromosome aberrations in Human lymphocytes *in vitro*. The test substance was tested in the presence and absence of metabolic activation (S9 mix prepared from Aroclor 1254 induced male Sprague-Dawley rat liver). The test article was dissolved in culture medium (MEM). Duplicate cultures of cells were exposed to the test substance for 4 hours in the presence of metabolic activation with cell harvest after 16 and 26 hours expression as well as for 20 hours in an experiment without S9-mix. The concentrations ranged between 78.13 – 625 µg/ml without metabolic activation and 156.25 – 1250 µg/ml with metabolic activation for 20 h harvest or 312.5 - 1250 µg/ml with metabolic activation for 30 h harvest.
Appropriate negative and positive controls were included.

Results

Precipitation was observed on the microscopic slides prepared from most of the cultures treated with the test substance. Mitosis was almost completely inhibited at dose levels >625 µg/ml in the absence of S9-mix, while in the presence of metabolic activation mitosis was severely reduced at 2500 and 5000 µg/ml. According to the laboratory evaluation criteria 625 µg/ml was selected as the highest concentration for evaluation without metabolic activation and 1250 µg/ml for assessment with S9-mix.
Henna Rot induced a dose-related decrease in the mitotic index with and without S9-mix at the 20 h harvest, while at the 30 h with S9-mix, mitosis was inhibited at all three dose-levels but without a clear concentration-effect-relationship.
There was an increase in the frequency of cells with structural chromosome aberrations (excluding gaps) in both of treatment groups with S9-mix at the highest dose level. No clastogenic potential was observed at any concentration when tested without metabolic activation. No increase in the numbers of polyploid cells was noted at any dose level in any assay.
Positive controls led to increased frequencies of cells with structural aberrations.

Conclusion
Under the conditions of the assay described, Henna rot (batch 830.72) induced an increase in structural chromosome aberrations in cultured human lymphocytes in the presence of S9-mix. Thus, Henna Rot is considered as weakly clastogenic.

Ref.: 27 (subm. II)

Sister chromatid exchange (SCE) test in Chinese hamster ovary (CHO) cells

Guideline:	OECD 479 (1986)
Test system:	Chinese hamster ovary (CHO) cells
Replicates:	duplicate cultures
Test substance:	Henna rot
Batch:	830.72
Concentrations:	25 – 200 µg/ml without metabolic activation (3 and 24 h exposure) 200 – 800 µg/ml with metabolic activation (3 h exposure)
Solvent:	DMSO
GLP:	Yes

Henna Rot was examined for genotoxic effects in the in vitro SCE test with CHO cells. The test was performed with and without metabolic activation (S9 mix from the liver of Aroclor 1254 induced rats). The test substance was dissolved in DMSO. Prior to the main study the cytotoxicity was evaluated in concentrations ranging from 50 – 1000 µg/ml. Based on the observed toxicity, concentrations in the range of 25 – 200 µg/ml without metabolic activation for the 3 and 24 h exposure periods and between 200 – 800 µg/ml with metabolic activation after 3 h of exposure were selected. After treatment cells were cultured in the presence of 5´-bromodeoxyuridine (BrdU) for further 24 hours. Thereafter, colcemid was added for a period of 2 – 3 h before end of incubation time and cells harvest. The solvent DMSO was used as negative control, while ethylmethane sulphonate (100 and 200 µg/ml) was used as a positive control without metabolic activation and cyclophosphamide (1 and 2 µg/ml) with metabolic activation.

Results

Cytotoxicity (about 50 % inhibition of mitosis) was observed in cultures treated with the highest concentrations in the presence or absence of S9-mix. No treatment-related increase in the frequencies of SCEs occurred at any concentration or exposure period.
The positive controls showed an increase in SCE demonstrating the sensitivity and validity of the test system.

Conclusion
Henna rot (batch 830.72) did not induce SCE in CHO cells in the presence or absence of metabolic activation under the experimental conditions used.

Ref.: 8 (subm. II)

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mouse Bone Marrow Micronucleus Test

Guidelines:	OECD 474 (1981)
Species/strain:	Mouse/CD-1
Group size:	5 male and 5 females per group
Test Substance:	Henna rot
Batch:	830.72
Dose level:	300 mg/kg bw
Solvent:	1% aqueous carboxymethylcellulose (CMC)
Route:	i.p.
Exposure period:	once
GLP:	Yes

The ability of Henna Rot to cause chromosomal damage *in vivo* was investigated in the mouse bone marrow micronucleus test. The choice of dose level was based on an initial range-finding study in which Henna Rot, formulated in 1% aqueous CMC was administered via intraperitoneal injection (i.p.). The test substance was administered to groups of 2 male and 2 female CD-1 mice once in doses ranged between 300 – 5000 mg/kg bw (i.p.) or 5000 mg/kg bw (oral). Doses at and above 500 mg/kg bw were lethal and evoked severe clinical findings.

Therefore, a single dose level of 300 mg/kg bw was selected and 5 male and 5 female mice were chosen for the main study.
A vehicle control (1% aqueous CMC) and a positive control (Cyclophosphamide, 50 mg/kg bw, i.p.) were also tested. Following dosing the animals were examined regularly and any mortality or clinical signs of reaction to the test compound were recorded. The mice were killed 24, 48 and 72 hours after administration. Bone marrow for micronuclei examination was prepared and after staining of the preparations 1000 polychromatic erythrocytes (PCE) were evaluated per animal and investigated for micronuclei. The ratio of polychromatic (PCEs) to normochromatic (NCEs) erythrocytes was determined to determine inhibition of erythropoesis.

Results
Clinical signs of lethargy, ptosis, diarrhoea and emaciation but no mortality were noted. Mice treated with Henna Rot showed no increase in the incidence of micronucleated PCEs when compared to the concurrent vehicle control group. A significant change in the NCE/PCE ratio

was observed, indicating cytotoxicity of bone marrow cells. The positive control exhibited increased numbers of micronucleated PCEs.

Conclusion
Henna Rot (batch 830.72) did not induce micronuclei in the bone marrow of mice treated with 300 mg/kg bw, a dose shown to cause clinical signs and bone marrow toxicity. Thus, there was no indication for clastogenic potential of Henna Rot expressed *in vivo* in this test.

Ref.: 31 (subm. II)

In vivo UDS test with rat liver cells

Guidelines:	OECD 486 (1997)
Species/strain:	Rat/Wistar
Group size:	3 male animals per dose
Test Substance:	Henna (*Lawsonia inermis*, COLIPA n° C169)
Batch:	1271 (1.36% 2-Hydroxy-1,4-Naphthoquinone)
Dose level:	1000 and 2000 mg/kg bw
Solvent:	Corn oil
Route:	oral (gavage)
Exposure period:	2 and 16 hours
GLP:	Yes

Henna Rot was assessed for its potential to induce DNA-damage and -repair in the in vivo UDS test using rat hepatocytes. The choice of dose level was based on an initial range-finding study. The highest dose was in line with the requirement for the top dose according to the current guidelines. The application volume was 10 ml/kg bw. After the treatment periods, the animals were sacrificed and liver perfusion was carried out. From each animal at least three primary hepatocytes cultures were established and exposed for 4 hours to 3H-thymidine. The net nuclear grain counts were determined by counting two slides per animal and 50 cells per slide.
Appropriate positive controls (N'N-dimethylhydrazinedihydrochloride at 40 mg/kg bw mg/kg bw for the 2 hour preparation interval and 2-acetylaminofluorene at 100 mg/kg bw for the 16 hour preparation interval) were used.

Results
Slight clinical findings in form of reduced activity and ruffled fur were observed in the treated rats. The viability of the hepatocytes was not significantly reduced. Treatment with 1000 or 2000 mg/kg bw of Henna Rot did not induce UDS in the hepatocytes of the treated animals. The positive controls gave the expected genotoxic effect.

Conclusion
Under the experimental conditions reported, the test substance did not induce DNA-damage, i.e. no increased repair synthesis in the hepatocytes of the treated rats.
Therefore, Henna Rot (*Lawsonia inermis*, batch 1271) was shown to be non-genotoxic in this *in vivo* UDS test when tested up to the highest dose level recommended by the guidelines.

Ref.: 24 (subm. II)

Overall conclusion on mutagenicity/genotoxicity

In previous investigations, Henna Rot (*Lawsonia inermis,* batch 830.72) induced structural chromosome aberrations in cultured human lymphocytes and gene mutations in the $TK^{+/-}$ mammalian cell gene mutation assay with mouse lymphoma cells. Two new genotoxicity tests with the batch 1271 (containing 1.36% 2-Hydroxy-1,4-Naphtochinon) led to negative results in the $TK^{+/-}$ mammalian cell gene mutation assay with mouse lymphoma cells and in an *in vivo* UDS test with rat hepatocytes. However, to exclude a clastogenic potential of Henna rot (*Lawsonia inermis*) additional testing with batch 1271 is required. An additional *in vitro* chromosome aberration test or (preferentially) an *in vitro* micronucleus test should be performed. In case of a positive result appropriate *in vivo* genotoxicity testing has to be considered.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

The product Henna Rot was administered by daily gavage to 100 pregnant female Sprague Dawley rats on day 6 through 15 of gestation at the dose levels of 40, 200 and 1000 mg/kg/day body weight and at a constant dose volume of 10 ml/kg/day according to the OECD N°414 (1981). A control group was administered with the vehicle alone, a 0.5 % aqueous solution of methylcellulose. Pregnant animals were killed on day 20 of gestation; macroscopic examinations of the dams were performed, visceral and skeletal malformations were recorded on the foetuses. No clinical signs, no abortions and no mortalities were recorded in any female of any group during the study. However, a very slight but significant decrease of body weight gain and food consumption was observed with the dams receiving 1000 mg/kg/day and was considered by investigators treatment-related. Pre and post-implantation loss, foetal body weight and sex-ratio were similar between control and all treated groups. At the external examination, no treatment-related anomalies or malformations were observed. In the highest dose group, two foetuses revealed dilatation of cerebral ventricles (lateral ones or 3rd one) and one foetus revealed cleft palate. At the skeletal examinations, reduced ossification of the pubic bone and cleft palate in one foetus were noted in the 200 mg/kg/day group; a significant reduced ossification of caudal vertebra and unossification of the 5th sternebra and the caudal vertebra, an increase in reduced ossification of the 1st to 4th metatarsals and of the pubic bone were noted in the 1000 mg/kg/day group. These foetal findings recorded at 200 and 1000 mg/kg/day were considered by the investigators to be probably treatment-related.

Under the experimental conditions adopted, the NOAEL of the test product, Henna Rot, was established at 200 mg/kg/day for the pregnant female rats and at 40 mg/kg/day for the rat foetuses.

Ref.: 12 (subm. I)

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

A case report survey between 1984 and 1989 showed that oral poisoning by a mixture of *Lawsonia inermis* dye and para-phenylenediamine dyes caused hospitalization of 31 Sudanese children. Mortality was high among the children and in total, 13 deaths occurred within 24 hours of supply to the hospital. However, it was para-phenylenediamine that was determined by the authors to be responsible for the adverse effects.

Ref.: 33 (subm. II)

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

Not applicable

3.3.14. Discussion

Marketed Henna represents a natural material derived from dried and powdered leaves of the plant *Lawsonia inermis*. Henna is used as a hair dye, based on the staining properties of one of its constituents, e.g. Lawsone. Modified Henna products, such as Black Henna are also available to consumers. The content of Lawsone among various modified Henna products may vary significantly, but these products contain some other substances for modifying the intensity of the colour provided by Henna alone. Aqueous pastes of Henna are also used for skin decoration. The present opinion only refers to the use of Henna as hair dye.

Toxicity
The calculated median lethal dose was > 2000 mg/kg bw (acute oral and dermal).
The NOAEL (No-Observed-Adverse-Effect-Level) of Henna Rot was 40 mg/kg bw (13 week day rat study); the NOAEL was 200 mg/kg/day for the pregnant female rats and 40 mg/kg/day for the rat foetuses (teratogenicity study).

Irritation, sensitisation

No separate skin irritation study was performed in experimental animals. However, *Lawsonia inermis* showed no irritative potential for the skin after a single occlusive application for 24 hours, when tested for acute dermal toxicity (described above) under enforced conditions.
Lawsonia inermis is slightly and transiently irritating to the eyes.
The results of a Buehler test suggest that *Lawsonia inermis* exhibited no potential to induce dermal sensitization in Guinea pigs under the conditions used. However, skin staining may have compromised evaluation.
There was no indication for any irritative or sensitizing potential under the conditions of RIPT study in Human volunteers.

Percutaneous absorption
The cutaneous application of the aqueous *Lawsonia inermis* pulp spiked with [14C]-Lawsone to mimic human use conditions onto the skin of male and female rats led to a percutaneous absorption of 0.2% determined as radioactivity after 72 hours, corresponding to an absolute absorption of 1.70 µg /cm². However, the exposure time in this experiment was only 40 minutes, whereas the actual use duration will be up to 2 hours.

Mutagenicity
In previous investigations, Henna Rot (*Lawsonia inermis*, batch 830.72) induced structural chromosome aberrations in cultured human lymphocytes and gene mutations in the $TK^{+/-}$ mammalian cell gene mutation assay with mouse lymphoma cells. Two new genotoxicity tests with the batch 1271 (containing 1.36% 2-Hydroxy-1,4-Naphthochinon) led to negative results in the $TK^{+/-}$ mammalian cell gene mutation assay with mouse lymphoma cells and in an in vivo UDS test with rat hepatocytes. However, to exclude a clastogenic potential of Henna rot (*Lawsonia inermis*) additional testing with batch 1271 is required. An additional in vitro chromosome aberration test or (preferentially) an in vitro micronucleus test should be performed. In case of a positive result appropriate in vivo genotoxicity testing has to be considered.

4. CONCLUSION

The SCCP is of the opinion that the information submitted is insufficient to assess the safe use of the substance as a hair dye.

To exclude a clastogenic potential of Henna Rot (*Lawsonia inermis*), additional testing with batch 1271 is required. An additional *in vitro* chromosome aberration test or (preferentially) an *in vitro* micronucleus test should be performed. In case of a positive result, appropriate *in vivo* genotoxicity testing has to be considered.

The traditional and current expanding use of Henna Rot (*Lawsonia inermis*) as a body-paint has not been assessed.

5. MINORITY OPINION

Not applicable

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22. Annex: “Produktspezifikation für Lawsonia Folium” “Plant preparations used as ingredients of cosmetic products”

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