Scientific Committee on Consumer Safety

SCCS

OPINION ON

Lawsonia inermis (Henna)

COLIPA n° C169

The SCCS adopted this opinion at
its 3rd plenary meeting on 19 September 2013

Corrigendum of 12 November 2021

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Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
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In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

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The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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Corrigenda made in the Opinion:
Page 19: Percutaneous absorption in vitro. DAp of 4.08 % to be used for MoS calculation.
Page 22: NOAEL of 2 mg/kg bw/d will be used for the MoS.
Page 37: Revision of the calculation of the Margin of Safety (MoS) from 210 to 156.
Page 39: Discussion on dermal absorption: DAp revised from 5.3% to 4.08%.

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1. BACKGROUND

The Cosmetics Directive[1] foresees a phasing-out of animal testing for cosmetic products. A ban of animal testing of finished cosmetic products has been in force since September 2004 and a testing ban on ingredients or combinations of ingredients since March 2009. As from March 2009, it is also prohibited in the EU to market cosmetic products and their ingredients which have been tested on animal in order to meet the requirements of the Directive, irrespective of the origin of these products. This marketing ban applies to all but the most complex human health effects to be tested to demonstrate the safety of cosmetic products (repeated-dose toxicity including skin sensitisation and carcinogenicity, reproductive toxicity and toxicokinetics), for which the legislator extended the deadline to March 2013.

Article 4a (2.4) of the Cosmetics Directive provides that Member States can request the Commission to grant a derogation from these provisions. Derogation shall only be granted if (a) the ingredient is in wide use and cannot be replaced by another ingredient able to perform a similar function and if (b) the specific human health problem is substantiated and the need to conduct animal tests is justified and supported by a detailed research protocol proposed as the basis for the evaluation. It is foreseen that the SCCS must be consulted prior to such derogation being granted by the Commission.

The Commission has received the first request for such derogation from the French authorities. The derogation request relates to Henna (*lawsonia inermis*) (CAS n. 84988-66-9). *Lawsonia inermis* is a natural material derived from powdered dried leaves of the Lawsonia inermis plant. Lawsone is the main active ingredient in henna (*lawsonia inermis*). Based on the staining properties of lawsone, it is mainly used as a hair dye, but also applied to the body. Lawsone corresponds to 2-hydroxy-1,4-naphthoquinone (CAS n. 83-72-7) and is present at 1 to 2% in the dried leaves of the plant. A hair dye formulation contains a maximum of 20% Henna powder suspended in 80% water.

The SCCS has evaluated the safety of henna and lawsone several times. However doubts remained in relation to the genotoxicity.

Opinions on lawsone:

- SCCNFP/0385/00, final of 13 March 2001
- SCCSNFP/0561/02, final of 27 February 2002
- SCCSNFP/0583/02, final of 17 September 2002
- SCCNFP/0798/04 of 16 February 2004. The conclusion in this opinion was that "*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*".

Opinions on henna:

- SCCNFP/0505/01, final of 17 September 2002.
- SCCP/0943/05 of 13 December 2005. The conclusion of this opinion was that: "*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

[1] Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products, OJ L 262, 27.9.1976, p. 169.

(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" (emphasis added).

In addition to the data that was relied on by the SCCS in the opinions above, additional data has become available since then.

The French authorities have carried out an *in vitro* mammalian cell gene mutation test in L5178Y TK mouse lymphoma cells and have obtained positive *in vitro* results with and without metabolic activation. They consider that these positive results require confirmatory *in vivo* testing. The French authorities therefore request a derogation from the animal testing ban in order to perform an *in vivo* test following the protocol OECD 474 Mammalian Erythrocyte Micronucleus Test, a test method that is included in the Test Methods Regulation[2] as 'B.12. Mutagenicity – *in vivo* Mammalian Erythrocyte Micronucleus Test' (the test is proposed to be carried out on batch 1271).

The company Logocos has initiated two *in vitro* tests, one micronucleus test with Chinese hamster V79 cells (OECD 487) that was positive and one micronucleus test with human lymphocytes (OECD 487) that was positive in the presence of metabolic activation and negative without it.

Reference is made to the above referenced SCCS opinions, the French derogation request and the data supplied by Logocos.

2. TERMS OF REFERENCE

With a view to the request for a derogation from the testing ban by the French authorities in order to carry out *in vivo* testing, the SCCS is asked to answer the following questions:

1. *Does the SCCS consider that the safe use of henna (Lawsonia inermis) in cosmetics, especially as a hair dye, can be established based on the available scientific data? Would the SCCS suggest concentration limits for the use of henna (Lawsonia inermis) in cosmetic products?*

2. *In case the safe use of henna (Lawsonia inermis) cannot be established based on the available scientific data, does the SCCS consider that the proposed in vivo testing following test guideline OECD 474 on batch 1271 is necessary and appropriate to obtain the data that will allow a conclusive safety assessment?*

3. *In addressing the necessity and appropriateness of the proposed in vivo test, could the SCCS in particular address the following questions:*

    - *is there a need for confirmatory animal testing given that there is already animal data available from two earlier in vivo tests?*
    - *could the additional required data be obtained based on non-animal approaches?*
    - *will the proposed testing of batch 1271 provide the necessary data to allow a subsequent safety assessment of henna (Lawsonia inermis)?*

[2] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), OJ L 142, 31.05.2008, p. 1 ff.

Supporting documents

1. Derogation request French authorities January 2011, cover letter
2. Derogation request French authorities January 2011, Note
3. Derogation request French Annex I – Study Report *in vitro* mammalian cell gene mutation test in L5178Y TK mouse lymphoma cells
4. Derogation request French Annex II – Validation of the analytical method for the determination of lawsone in dimethylsulfoxide concentrations
5. Derogation request French Annex III – Evaluation of aqueous henna extraction process
6. Follow-up letter Commission to French authorities March 2012
7. Reply French authorities to Commission
8. Letter Logocos 30 August 2011
9. Letter Logocos 30 August 2011, Attachment: Study Report, Induction of micronuclei in cultured human peripheral blood lymphocytes
10. Letter Logocos 26 September 2011
11. Letter Logocos 26 September 2011, Attachment: Study Report *in vitro* Micronucleus test in Chinese Hamster V 79 cells
12. Dossier Update of Lawsonia Inermis (Henna, Colipa Nr. C169), August 2006

3. OPINION

3.1 Chemical and Physical Specifications

Taken from SCCP/0943/05

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 / EC number

CAS: 84988-66-9
EC: 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

Powdered leaves of Lawsonia inermis plant is marketed as Henna. The natural constituents of *Lawsonia inermis* are essential oils, 1,4-naphthoquinone, tannins, gallic acid, flavonoids, terpenoids, lipids, sugars, triacontyl tridecanoate, mannitol, xanthones, coumarins (5-allyloxy 7-hydroxycoumarin), 2-3% resins, 5-10% tannic constituents and 0.5 - 2% Lawsone (2-hydroxy-1,4-naphthoquinone, CAS No. 83-72-7). A major portion of Lawsone is glycosidic bound, and that is cleaved by enzymatic hydrolysis of the glycosidic hennosids and auto-oxidation of aglucons.

Ref.: subm. III (Dossier update on *Lawsonia inermis)*

Taken from SCCP/0943/05

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%

Ref.: 2 (subm.I
21 (subm. II)

In the dossier of submission III, general specification for *Lawsonia inermis* is described as: Loss on drying: <5%, Total ash >15%, Lawsone content <2%, Flavonoid content <1%, Carotenoids <1 ppm, Water soluble extract ~5%. However, supporting documentation is not provided for the general specification.

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

SCCS Comment
Following an inquiry of the SCCS with regard to the analytical specification of Henna the raw data were checked by the applicant and a transfer error from the raw data to the certificate analysis and consequently to the dossier was detected. The applicant re-analysed a backup sample of the batch 1271. The Lawsone content of 1.28% was confirmed and the water soluble extract was determined by means of the Ph. Euro. 4.0 method which according to the applicant is very similar to the DAC method used for the batch 830.72. The value for the water soluble extract was 27% and considered similar to that of batch 830.72. The water soluble extract was not fully chemically characterised.

In submission II from 2002, a further batch 878.03 was characterized, the water soluble extract was 34%. In submission I from 1999 a similar value (> 30%) was given in the product specification file (subm. I, ref. 25).
Altogether, it is concluded that the different batches of Henna do not vary considerably with regard to the water soluble extract. Since also the Lawsone content is comparable the results of the toxicological studies of these different batches can be used together for the assessment of Henna.

Ref.: AR4

In submission I, batch 830.72 was not properly characterized, which caused problems with the interpretation of especially the mutagenicity studies. In the submission II 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)

There is an additional batch (20202207) used in 2 genotoxicity tests in submission IV.
Henna Extract 10%, batch number 20202207, was a brown powder. It was stored at 15-25 °C protected from light. Photometrical content was 10% (content of the main active ingredient 2-Hydroxy-1,4-Naphthochinon with a molecular weight of 174.15 g/mol, also known as Lawsone). The ash content was 8.6%. Test article stock solutions were prepared by formulating Henna Extract 10% under subdued lighting in purified water, with the aid of vortex mixing, warming at 37 °C and ultrasonication, to give the desired test concentrations.

Ref.: Covance 2011 (submission IV, appendix 5)

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 (subm. I)

In the dossier of submission III of *Lawsonia inermis,* general specification of impurities is described as:
Impurities of heavy metals and pesticides are not higher than in the current version of the respective directive (details can be found in references 23 and 14 of submission III).

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 Pow)

Log P_{ow}: /

3.1.8 Additional physical and chemical specifications

Melting point:	/
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm):	/

3.1.9 Homogeneity and Stability

Lawsonia inermis is stable after harvest and powdering for at least 2 years at room temperature and protection of light.

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)

SCCS Comment
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.

In submission IV (Henna powder: evaluation of aqueous henna extraction process. Study number 35943 AHS), APPENDIX Ill (Study performed by CIT), Lawsone is determined in Henna extracts prepared by mixing 10 g Henna with 40 ml water. Thus the concentration of Lawsone in aqueous Henna extracts used for the mutagenicity testing may be lower than that under real exposure conditions where 10 g Henna powder is mixed with 30 ml water.

3.3 Toxicological Evaluation

Taken from SCCP/0943/05

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 (Wistar Rat).

Lawsonia inermis showed no irritative potential for the skin after a single occlusive application for 24 hours to Wistar Rats, 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

A volume of 0.1 ml of the 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.: 4 (subm. I
Ref.: 28 (subm. II)

3.3.3 Skin sensitisation

Buehler Test

Guideline:	OECD 406
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
Purity:	/
Route:	Occlusive epicutaneous induction (50%) and challenge (50%)
Carrier:	Petrolatum
GLP:	Yes
Date:	1990

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 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) 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 patch 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 irritant or sensitizing potential under the conditions of the study in Human volunteers.

Ref.: 13 (subm. II)

SCCS Comment
Such studies are not considered ethical by the SCCS.

Experience under specific conditions in humans

It is known from the literature that *Lawsonia inermis* is widely used both as a hair dye and for skin staining. 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)

A current review of the dermatological safety of Henna is available. The following table is extracted from the review and illustrates possible cases of contact allergy to Henna.

Year	No.	Sex	Age (years)	Clinical data	Positive patch test reactions	Comments
2011	1	F	3	Bullous eruption 1 day after applying henna paste to the hand	Pure henna powder paste 10% pet.; PPD negative	The paste was made of dried leaves of the henna plant with no additions of any kind; no control tests performed
2009	1	F	30	Erythema and oedema of the leg 1 week after applying henna in vinegar	Henna powder 1% in saline and in vinegar; negative to vinegar and PPD	No control tests performed
2008	1	F	60	Acute bilateral palpebral dermatitis after applying henna paste to the hair	Henna powder 10% pet.; PPD negative	The patient had had a similar reaction to henna 1 year earlier; no additives were used; no control tests performed
2006	1	M	9	Two weeks after a black henna tattoo, generalized dermatitis developed	Lawsone 10% pet.; also PPD, IPPD, and benzocaine	Later, the patient had allergic contact dermatitis caused by permanent, non-henna hair dye; the allergy to lawsone was considered to be provoked by the reaction to PPD; no control tests performed
2003	1	F	50	Three episodes of acute palpebral dermatitis after dyeing hair with henna	Henna powder 10% pet. and tlawsone 5% pet.; PPD negative	20 controls were negative to henna 10% pet. and to lawsone 5% pet.
2002	1	M	26	Within 2 weeks after a henna tattoo, lichenoid dermatitis developed	Henna powder pure, 10% aqua and 20% aqua positive; PPD and black henna preparation positive	The natural henna powder did not contain PPD (mass spectrometry); 'control tests' were negative
2001	1	M	26	Dermatitis 14 days after a black henna tattoo containing PPD	PPD; natural henna powder 10% and 20% aqua	It was not ascertained that the 'natural' henna powder used for patch testing did not contain PPD; no control tests performed
2001	1	F	9	Two days after a second tattoo on day 7, dermatitis appeared at the application site	PPD and natural henna (not stated how tested); commercial henna was negative	No control tests performed
2001	1	F	38	Oedema of the face and tongue within 4 hr after applying commercial henna hair dye	PPD, commercial henna 1% and natural henna 1% in saline	No control tests performed; it was not ascertained that the natural henna did not contain PPD
2000	1	M	35	Ten days after a black henna tattoo, dermatitis developed	PPD; unrelated henna powder 10% pet. positive; another pure henna sample 1% aqua negative	It was not checked whether the unrelated henna powder contained PPD; no control tests performed
2000	1	M	38	Twenty days after a henna non-permanent tribal tattoo, lichenoid dermatitis developed	PPD, henna (ground dry leaves in pet.)	No control tests performed with henna; unknown whether the tribal tattoo contained PPD
1999	2	F	22, 52	Dermatitis after traditional dyeing of the hands with (plain) henna	Henna ground leaves in pet. jelly (test concentration unknown) in 1 after 2 days; PPD was not tested; the other patient was not tested	Both patients had previous episodes of allergic contact dermatitis caused by plain henna; no control tests performed
1997	1	F	33	Ectopic dermatitis on the eyelids, cheeks and forehead after application of henna to the nails on various occasions; the dermatitis stopped and did not recur after cessation of the use of henna	Henna dye 2% aqua, nickel; reaction to PPD not mentioned	No control tests performed
1997	1	F	30	Dermatitis of the face and scalp after applying an aqueous paste of natural henna to the hair on two occasions, starting within hours	Pure powdered henna 10% pet.; nickel; negative to PPD; open test with henna negative after 30 min	10 healthy controls were negative
1992	1	M	69	Recurring dermatitis on the face and oedema of the eyelids from using a sunscreen cream	Sunscreen cream; lawsone 5% pet. (twice);CI 12 150 (Solvent Red 1); CI 12 010 (Solvent Red 3)	Lawsone was negative in 10 controls; the colours were both ingredients of the sunscreen cream, but the source of sensitization to lawsone remained unknown
1988	1	M	19	Acute swelling, oedema, itching and burning within 2 h after applying a paste of fresh henna leaves ground in water	Paste of fresh henna leaves ground in water; commercial henna (test concentration not mentioned)	No control tests performed
1986	1	F	13	Recurring dermatitis after applying commercial henna powder or a paste of fresh henna leaves to the backs of the hands	Strongly positive to commercial henna, henna leaves made into a paste, and lawsone	No test concentrations mentioned; no control tests performed
1980	1	F	46	Dermatitis of the right index finger spreading to other fingers after applying henna to the hands of her husband; the patient had several previous episodes of dermatitis of her hands after applying henna on them	Repeatedly positive to henna aqueous paste; positive to extracts of henna powder	Three controls with aqueous extracts of henna were negative

Ref.: AR3

SCCS Comment

There are rare case reports of contact allergy to Henna in the literature.

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 analysed 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 radio-labelled material.

Study 1, percutaneous absorption *in vitro* using pig skin

Guidelines:	/
Test system:	Isolated pig skin
Method:	Permeation chambers (flow through system)
Number of chambers	6
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
Date	1992

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 analysed 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 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)

SCCS Comment
Exposure time was only 30 min. No details of the single chamber values were provided.

Study 2, percutaneous absorption *in vitro* using human skin

Guideline	/
Chambers	Teflon diffusion cells
Surface area	0.64 cm^2
Membrane	from frozen human abdominal skin
Donors	see individual results
Membrane thickness	200-300 µm
Membrane integrity	Tritiated water
Substance	^{14}C- Lawsone (93-97% radiopure)
Samples	sample A Commercial Henna powder
	Sample B Commercial Henna powder
	Sample C Commercial Henna-containing shampoo
	Sample D Commercial Henna-containing shampoo

Dose:	Sample A 3 mg powder per cell (or 13 mg paste) Sample B 3.9 mg powder per cell (or 13.9 mg paste) Samples C & D; 1.2 mg shampoo
Receptor	Hank's balanced solution; pH 704; flow 1.5 mL/hr
Observation:	6 hourly intervals for 24 hours (with extension to 72 hours)
Solubility	/
Stability	/
Analysis	liquid scintillation counting
GLP	compliant
Date	published 2007

A: The dose of [^{14}C]-lawsone applied in the henna paste was about 0.21 ±0.03 µCi (1.51 ± 0.22 µg/cm^2) in the 24-hour studies and 0.19 ± 0.004 µCi (1.36 ± 0.03 µg/cm^2) in the 72-hour studies as determined from aliquots taken from the paste.

B:The dose of [^{14}C]-lawsone applied was approximately 0.23 ± 0.001 µCi (1.65 ± 0.01 µg/cm^2) in the 24-hour studies and 0.13 ± 0.01 mµCi (0.93 ± 0.04 µg/cm^2) in the 72-hour studies.

The actual dose of [^{14}C]- lawsone applied to each cell was about 0.15 ± 0.01 µCi (1.08 ± 0.07 µg/cm^2) (product C) and 0.14 ± 0.04 µCi (1.00 ± 0.29 µg/cm^2) (product D) in the 24-hour studies and
0.11 ± 0.02 µCi (0.79 ± 0.14 µg/cm^2) (product C) and 0.09 ±0.003 µCi (0.65 ± 0.02 µg/cm^2) (product D) in the 72-hour studies.

[^{14}C]-Lawsone was added separately to each commercial product. The products remained on the skin for 5 min (shampoos) and 1 h (henna pastes).

Table 1 Penetration of lawsone from a commercial henna hair paste (product A) in 24 and 72 hours

	Applied dose penetrated (%)	
	24 hours[1]	72 hours[2]
Receptor fluid	0.3 ± 0.1^a	0.5 ± 0.1^a
Stratum corneum	1.6 ± 0.2^a	0.5 ± 0.1^a
Epidermis and dermis	0.6 ± 0.1^a	0.3 ± 0.03^a
Total in skin	2.2 ± 0.3^a	0.8 ± 0.1^a
Total penetration	2.5 ± 0.3^a	1.3 ± 0.1^a
Wash	102.0 ± 3.3	93.1 ± 2.2
Recovery	105.1 ± 3.1	94.5 ± 2.2

[1]Values are the mean ± SEM of eleven replicates (2 donors). Other 24-hour values are the mean ± SEM of six replicates (2 donors). Lawsone applied dose was $1.51 \pm 0.22\,\mu g/cm^2$.
[2]Values are the mean ± SEM of 5 replicates (1 donor). Lawsone applied dose was $1.36 \pm 0.03\,\mu g/cm^2$.
[a]Significantly different between the 24 and 72-hour values (t-test $p < 0.05$).

Table 2 Penetration of lawsone from a commercial henna hair paste (product B) in 24 and 72 hours

	Applied dose penetrated (%)	
	24 hours[1]	72 hours[2]
Receptor fluid	1.3 ± 0.3	1.6 ± 0.2
Stratum corneum	2.8 ± 0.3^a	1.6 ± 0.2^a
Epidermis and dermis	1.2 ± 0.2	1.3 ± 0.1
Total in skin	4.0 ± 0.4	2.9 ± 0.3
Total penetration	5.3 ± 1.0	4.5 ± 0.4
Wash	87.4 ± 3.0	72.7 ± 4.5
Recovery	93.3 ± 3.1	77.4 ± 4.4

[1]Values are the mean ± SEM of ten replicates (2 donors). Other 24-hour values are the mean ± SEM of five replicates (2 donors). Lawsone applied dose was $1.65 \pm 0.01\,\mu g/cm^2$.

[2]Values are the mean ± SEM of five replicates (1 donor). Lawsone applied dose was $0.93 \pm 0.04\,\mu g/cm^2$.

[a]Significantly different between the 24 and 72-hour values (t-test $p < 0.05$).

Table 3 Penetration of lawsone from a commercial henna shampoo (product C) in 24 and 72 hours

	Applied dose penetrated (%)	
	24 hours[1]	72 hours[2]
Receptor fluid	0.3 ± 0.02^a	0.4 ± 0.04^a
Stratum corneum	2.4 ± 0.3^a	1.4 ± 0.2^a
Epidermis and dermis	1.2 ± 0.2^a	0.5 ± 0.1^a
Total in skin	3.6 ± 0.5^a	1.9 ± 0.2^a
Total penetration	3.9 ± 0.5^a	2.3 ± 0.3^a
Wash	90.3 ± 3.0	82.0 ± 3.2
Recovery	94.7 ± 3.1	84.7 ± 3.4

[1]Receptor fluid values are the mean ± SEM of twenty-four replicates (3 donors). Other 24-hour values are the mean ± SEM of twelve replicates (3 donors). Lawsone applied dose was $1.08 \pm 0.07\,\mu g/cm^2$.

[2]Values are the mean ± SEM of twelve replicates (3 donors). Lawsone applied dose was $0.79 \pm 0.14\,\mu g/cm^2$.

[a]Significantly different between the 24 and 72-hour values (t-test $p < 0.05$).

Table 4 Penetration of lawsone from a commercial henna shampoo (product D) in 24 and 72 hours

	Applied dose penetrated (%)	
	24 hours[1]	72 hours[2]
Receptor fluid	0.3 ± 0.04^a	0.6 ± 0.1^a
Stratum corneum	4.5 ± 0.04	2.4 ± 0.2
Epidermis and dermis	2.3 ± 0.6	0.6 ± 0.2
Total in skin	6.8 ± 1.4	3.0 ± 0.4
Total penetration	7.2 ± 1.4	3.5 ± 0.4
Wash	83.0 ± 2.9	92.6 ± 0.5
Recovery	90.5 ± 2.0	96.3 ± 0.3

[1]Values are the mean ± SEM of 10 replicates (2 donors). Other 24-hour values are the mean ± SEM of seven replicates (2 donors). Lawsone applied dose was 1.00 ± 0.29 µg/cm^2.

[2]Values are the mean ± SEM of three replicates (1 donor). Lawsone applied dose was 0.65 ± 0.02 µg/cm^2.

[a]Significantly different between the 24 and 72-hour values (t-test $p < 0.05$).

Below it is the mean + standard error of the mean that is reported:

For the paste A:
2.5 ± 0.3% of the applied dose of Lawsone was considered to have penetrated (amount Lawsone applied 1.5 ± 0.22 µg/cm^2; 7 chambers, 2 donors).

For paste B:
5.3 ± 1.0% of the applied dose of Lawsone was considered to have penetrated (amount Lawsone applied 1.65 ± 0.01 µg/cm^2; 10 chambers, 2 donors).

For shampoo C:
3.9 ± 0.5% of the applied dose of Lawsone was considered to have penetrated (amount Lawsone applied 1.08 ± 0.07µg/cm^2; 24 chambers, 3 donors).

For shampoo D:
7.2 ± 1.4% of the applied dose of Lawsone was considered to have penetrated (amount Lawsone applied 1.00 ± 0.29 µg/cm^2; 10 chambers, 2 donors).

Ref.: 15 (subm. II)
Ref.: AR1

SCCS Comment
The exposure time in the Henna paste study (1 hour) did not comply with the exposure time used by consumers (up to 2 hours).
Only the dermal penetration of Lawsone was determined; other compounds present in Henna (see 3.1.4) were not measured.
The study report is a publication and all data were not reported in detail.

The SCCS considers that in principle for the MoS calculation the value of paste B (mean + 2SD) should be used adding the amounts in the receptor fluid and in the epidermis and dermis. Since no single values were reported and 2 donors were used, the SCCS will add two standard deviations (derived from the average SEM's) to the sum of the abovementioned amounts. Thus, th SCCS will use a dermal absorption (DAp) of 2.5% + (2 x 0.79) = 4.08% for the calculation of the MoS.

Study 3, 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 [^{14}C]-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%) [^{14}C]-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 [^{14}C]-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 [^{14}C]-Lawsone/animal or corresponding to 0.86 *Lawsonia inermis* spiked with [^{14}C]-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, faeces, 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 [^{14}C]-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 [^{14}C] was highest at 45 min after application and declined with an initial half-life of approximately 2 h. The [^{14}C]-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 [^{14}C] substance. The mean radioactivity levels of blood and the 14 analysed 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 [^{14}C]-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.

Ref.: 9 (subm. II)

SCCS Comment

The exposure time in the study (40 minutes) did not comply with the exposure time used by

consumers (up to 2 hours). Only the dermal penetration of Lawsone was determined; other compounds present in Henna (see 3.1.4) were not measured.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (28 days) oral toxicity

No data submitted

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

Guideline:	OECD 408 (1981)
Species/strain:	Sprague-Dawley rats Crl CD (SD) BR
Group size:	10 males + 10 females
Test substance:	Henna Rot suspended in 0.5 % aqueous methylcellulose solution
Batch:	830.72
Purity:	/
Dose:	0, 40, 200, 1000 mg/kg bw/d by gavage
GLP:	In compliance
Date:	June 1994 – May 1995

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.

Results

No mortality was observed during the study.

In the high dose group, 2/40 animals occasionally presented signs of poor clinical condition (loud breathing (1/20 males), piloerection (1/20 females)) and 6/20 males presented ptyalism from week 9 or 11 onwards. Brown urine was noted in all males and females (from week 9 onwards), accompanied by a brown tail in some animals (from week 11 approximately). All clinical signs were reversible after 4-weeks recovery period, except for brown-coloured tail.

Mean food consumption was similar to that of the respective controls. Mean body weight gain of the treated males was 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 controls resulting in mean absolute body weight values statistically significantly different from that of controls (from week 5 onwards) and lowered by -10% and -8%, respectively, at the end of the treatment period; this finding was not dose-related and was not considered by investigators to be treatment-related. Mean body weight gain in high-dose animals was similar to that of the controls during the recovery period.
Neither treatment-related ophthalmological abnormalities nor effects in clinical chemistry (blood biochemistry, urinalysis) were noted in any treated group.
Concerning haematological parameters, slightly lower (statistically significant) erythrocyte count (-7% for males and -8% for females) and haemoglobin (-6% for males and -5% for females) were noted in the high dose group when compared to the control values, these differences were considered of no toxicological importance by the investigators. These findings were reversible after the 4-week recovery period.

In the highest dose group, statistically significant higher mean relative kidney (+18% for males and +13% for females), relative spleen (+29% for males and +33% for females), relative liver (+10% for males) and relative testes weights (+15% for males) were noted. All these findings were reversible after the 4-week recovery period except for the relative kidney weight of the females (+13%), but this was considered to be of no toxicological importance by the investigators as no relevant microscopic findings were noted in these treated females. Also the increased liver and testes weights were considered to be of no toxicological importance by the investigators as no relevant microscopic findings were noted in these organs in treated males. The relative weight changes of the kidneys in males and of the spleen in both sexes were considered by the investigators to be treatment-related.

In the high dose group, the hair and/or extremities (5/10 females), as well as the fore-stomach (all animals) and the mucosa of the urinary bladder (1/10 males and 1/10 females) were coloured orange related to the staining properties of the test substance.
Concerning microscopic examinations, no findings of toxicological relevance were noted at the low dose level. 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 for both males and females. Haemosiderosis (mid and high dose groups) and extramedullary haemopoiesis (high dose group) were noted in the spleen and were considered to be treatment related for both males and females. Except for the haemosiderosis in the spleen and the dyeing effects in the high dose group, all findings were reversible during the recovery period.

Conclusion

Based on these results, the NOAEL (No-Observed-Adverse-Effect-Level) of Henna Rot was established to be 40 mg/kg bw/day.Ref.: 3 (subm. I)

SCCS comment
Because Henna Rot contains besides Lawsone several other ingredients, the results of this study will not be used for the calculations of the MoS. According to SCCNFP/0583/02, the NOAEL of Lawsone is 2 mg/kg bw/d. In the context of a genotoxicity study (AR6 -Kirkland 2003), a 90-day oral toxicity study in rats was performed: the authors considered the NOAEL for Lawsone to be 2–7 mg/kg bw per day.
The SCCS will use a NOAEL of 2 mg/kg bw/d for the calculation of the MoS.

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

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
Purity:	/
Solvent:	DMSO
Concentrations:	50, 100, 500, 1000, 2500 and 5000 µg/plate with and without metabolic activation
Treatment:	direct plate incorporation assay with 48 h incubation without

	and with S9-mix
GLP:	in compliance
Study period:	11 September 1990 – 15 October 1990

Henna Rot 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 TA98, TA100, TA1535, TA1537 and TA1538 were exposed to Henna Rot (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 an increase in 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 used, Henna Rot was not mutagenic in this gene mutation tests in bacteria.

Ref.: 15 (subm. I)
5 (subm. II)

Bacterial gene mutation assay

Guideline:	/
Test system:	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and streptomycin-resistant strains TA98strp, TA100strp, TA1535strp, TA1537strp, TA1538strp
Replicates:	triplicate plates, two independent assays
Test substance:	Henna Rot
Batch:	830.72
Purity:	/
Solvents:	DMSO and water
Concentrations:	50, 100, 500, 1000, 2500 and 5000 µg/plate with and without metabolic activation
Treatment:	direct plate incorporation assay with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	27 November 1991 – 28 January 1992

Henna Rot 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 TA98, TA100, TA1535, TA1537 and TA1538
- suspended in water and tested in streptomycin-resistant strains TA98 strp, TA100 strp, TA1535 strp, TA1537 strp and TA1538 strp
- suspended in DMSO and tested in streptomycin-resistant strains TA98 strp, TA100 strp, TA1535 strp, TA1537 strp and TA1538 strp

Appropriate negative and positive controls were included.

Results

No bacteriotoxic effect was observed but Henna Rot 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 an increase in the number of 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 in the number of revertants by the positive controls.

Conclusion

Under the experimental conditions selected, Henna Rot (batch 830.72) was not mutagenic in this gene mutation test in bacteria.

Ref.: 16 (subm. I)
6 (subm. II)

SCCS Comment

The sensitivity and validity of the test system used was demonstrated by the significant induction in the number of revertants by the positive controls.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 a 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 test (*hprt* locus)

Guideline:	OECD Guideline 476 (1984)	
Cells:	Chinese hamster V79 cells	
Replicates:	Two independent assays	
Test substance:	Henna Rot	
Batch:	830.72	
Purity:	/	
Solvent:	Water	
Concentrations:	first experiment:	1, 5, 10, 50, 100 and 200 µg/ml without S9-mix 10, 50, 100, 250, 500 and 1000 µg/ml with S9-mix
	second experiment:	10, 50, 100, 250, 500, 1000, 2000 and 3000 µg/ml with S9-mix
Treatment:	3 h treatment both without and with S9-mix; expression period 7 days and a selection period of 7 days	
GLP:	In compliance	
Study period:	29 June 1994 – 29 August 1994	

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 µg/ml with and without metabolic activation. Thereafter, two independent main assays were conducted using concentration ranges of 1 – 200 µg/ml without S9-mix and 10 – 1000 µg/ml or 10 – 3000 µg/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 concentration of 200 µg/ml without S9-mix and at of 2000 µg/ml with S9-mix. The test substance did not cause an 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-concentration-dependent increase to a maximum factor of 3.3 was observed at the lowest concentration only, while higher concentration levels showed values 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 increase in the mutant frequencies by the positive controls.

Conclusion

Under the test conditions used, Henna Rot (batch 830.72) is not mutagenic in the *hprt* gene mutation test with V79 cells.

Ref.: 12 (subm. I)
7 (subm. II)

Mammalian cell gene mutation test (*tk* locus)

Guidelines:	OECD 476 (1998)
Cells:	Mouse lymphoma cell line L5178Y
Replicates:	duplicate cultures in 2 independent experiments
Test Substance:	Henna Rot
Batch:	830.72
Purity:	/
Solvent:	Fischer's medium
Concentrations:	78.13, 156.25, 312.5, 625 and 1250 µg/ml both with and without S9-mix
Treatment:	3 h treatment both without and with S9-mix; expression period 48 h and a selection period of 10-14 days
GLP:	In compliance
Study period:	15 November 1991 – 4 February 1992

Henna Rot 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. S9-mix was obtained from the liver of male Wistar rats induced with Aroclor. Concentrations were based on the results of a preliminary toxicity test measuring survival. The main experiment was performed with and without S9-mix and a treatment period of 3 h. Appropriate negative and positive controls were included.

Results

In both experiments and both with and without metabolic activation a concentration dependent and statistically significant increase in the mutant frequency was observed. The positive controls showed a distinct increase in induced total mutant colonies.

Conclusion

Under the experimental conditions used Henna Rot was mutagenic in the *tk* gene mutation test with mouse lymphoma cells.

Ref.: 18 (subm. I)
26 (subm. II)

Mammalian cell gene mutation test (*tk* locus)

Guidelines:	OECD 476 (1998)	
Cells:	Mouse lymphoma cell line L5178Y	
Replicates:	Two independent experiments	
Test Substance:	Henna (*Lawsonia inermis*, COLIPA n° C169)	
Batch:	1271	
Purity:	/, (content of 2-Hydroxy-1,4-naphthoquinone 1.36%)	
Solvent:	Deionized water	
Concentrations:	Experiment I:	150, 300, 600, 900 and 1200 µg/ml without S9-mix 75, 150, 300, 600 and 900 µg/ml with S9-mix
	Experiment II:	78.1, 156.3, 312.5, 625 and 1250 µg/ml without S9-mix
Treatment:	Experiment I:	4 h treatment both without and with S9-mix; expression period 72 h and a selection period of 10-15 days
	Experiment II:	24 h treatment both without S9-mix; expression period 48 h and a selection period of 10-15 days
GLP:	In compliance	
Study period:	28 February 2005 – 25 April 2005	

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. Henna 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 Henna in the solvent (deionized water) was analysed in respect to Lawsone for a period of at least 4 hours. Appropriate negative and positive controls were included.

Results

Precipitation of Henna was observed at all concentrations. 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 metabolic activation and at 900 µg/ml and above with metabolic activation.
No biologically relevant and reproducible increase of the mutant frequency was observed in any of the experiments. An isolated positive response was seen after 4 hours treatment in the absence of S9-mix at 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 Henna (batch 1271, containing 1.36% 2-Hydroxy-1,4-naphthoquinone) is not mutagenic in the *tk* gene mutation test with mouse lymphoma cells.

Ref.: 23 (subm. II)

***In vitro* Mammalian Cell Gene Mutation Test (*tk*-locus)**

Guideline:	OECD 476 (1997)

Cells:	Mouse lymphoma L5178Y *tk*$^{+/-}$ cells	
Replicates:	duplicate cultures in two independent experiments	
Treatment	3 h treatment without and with S9-mix; expression period 48 h and selection period of 11-12 days	
GLP:	in compliance	
Study period:	25 August 2009 – 4 November 2009	
Test substance:	Henna powder	
Batch:	1271	
Purity:	/	
Content:	1.2 mg/ml Lawsome in Henna extract	
Solvent:	sterilised water	
Concentrations:	experiment 1:	0.078, 0.156, 0.313, 0.625, 1.25, 1.875, 2.5 and 3.75% v/v of Henna extract without S9-mix 0.039, 0.078 0.156, 0.313, 0.625, 1.25, 2.5 and 5% v/v of Henna extract with S9-mix
	experiment 2:	0.156, 0.313, 0.625, 0.9375, 1.25, 1.875, 2.5 and 3.75% v/v of Henna extract without S9-mix 0.039, 0.078 0.156, 0.313, 0.625, 1.25, 2.5 and 5% v/v of Henna extract with S9-mix
Reference substance:	Lawsone (2-hydroxy-1,4-naphthalenedione)	
Batch:	12091-199	
Purity:	98.8 %	
Solvent:	DMSO	
Concentrations:	experiment 1:	1.09, 2.19, 4.38, 8.75, 17.5, 26.3, 35 and 52.5 µg/ml of Lawsone without S9-mix 0.55, 1.09, 2.19, 4.38, 8.75, 17.5, 35 and 70 µg/ml of Lawsone with S9-mix
	experiment 2:	2.19, 4.38, 8.75, 13.125, 17.5, 26.25, 35 and 52.5 µg/ml of Lawsone without S9-mix 1.09, 2.19, 4.38, 8.75, 17.5, 26.3, 35 and 52.5 µg/ml of Lawsone without S9-mix

Henna powder and Lawsone (reference substance) were assayed for gene mutations at the *tk* locus of mouse lymphoma cells in both the absence and presence of S9 metabolic activation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Lawsone was dissolved in DMSO at 7 mg/ml, allowing testing of final concentrations of Lawsone equivalent to those present in the Henna extract, taking into account that the volume of treatment with DMSO was 1% (i.e. 200 µl/20 ml culture medium). Test concentrations were based on the results of a preliminary toxicity test on toxicity measuring relative suspension growth. Eight concentrations were tested. Cells were treated with 8 concentrations of Henna powder and Lawsone (reference substance) for 3 h (without and with S9-mix) and for 24 h (without S9-mix). After an expression period of 48 h, cells were harvested approximately one week after the end of the expression period.
In the main tests, cells were treated with 8 concentrations of Henna powder and Lawsone (reference substance) for 3 h followed by an expression period of 48 h to fix the DNA damage into a stable *tk* mutation. Approximately 12 days after the end of the expression period toxicity was determined. Toxicity was measured in the main experiments as percentage total growth of the treated cultures relative to the total growth of the solvent control cultures. To discriminate between large (>25% of the diameter of the well; indicative for mutagenic effects) and small colonies (<25% of the diameter of the well; indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results

Precipitation was not seen at the highest concentrations used of both Henna powder and Lawsone (reference substance). Without and with metabolic activation the required decrease in relative total growth obtained with Henna powder was observed (10-20% cell survival at the highest concentration); with Lawsone (reference substance), the required decrease was obtained with metabolic activation only.
Exposure to Henna powder resulted in a concentration-related and statistically significant increase in the mutant frequency exceeding the global evaluation factor of 126 x 10^{-6}.
Exposure to Lawsone (reference substance) with metabolic activation also resulted in a concentration-related and statistically significant increase in the mutant frequency exceeding the global evaluation factor of 126 x 10^{-6} , whereas without metabolic activation a biologically relevant increase in the mutant frequency was not observed.

Conclusion

Under the experimental conditions used Henna powder (without and with metabolic activation) and Lawsone (with metabolic activation) were mutagenic in this gene mutation test in mouse lymphoma cells.
As in this study the concentration ranges of the reference compound Lawsone were defined to be similar to the content of Lawsone in the concentrations of Henna powder, it was observed that without S9-mix, the mutagenic activity of the Henna extract is not directly related to the presence of Lawsone in the Henna extract. With S9-mix both Henna powder of Lawsone showed quite similar mutagenic activity suggesting that Lawsone when metabolised, is one component of the Henna extract responsible for the induction of mutation.

Ref.: CIT study (submission IV, app. I)

SCCS Comment
SCCS agrees that a 24 h exposure experiment was not performed as positive results were already found after 3 h treatment. Although colony sizing was mentioned in material and methods, results of colony sizing were not reported.

Chromosome aberration test in cultured human lymphocytes

Guideline:	OECD 473 (1981)
Cells:	Human lymphocytes
Replicates:	duplicate culture in a single experiment
Test substance:	Henna Rot
Batch:	830.72
Purity:	/
Solvent:	Culture medium
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
Treatment:	4 h treatment without S9-mix, harvest time 16 or 26 h after the end of treatment 24 h treatment with S9-mix, harvest time 24 h after the start of treatment
GLP:	in compliance
Study period:	13 January 1992 – 28 January 1992

Henna Rot was assessed for its potential to induce structural chromosome aberrations in Human lymphocytes *in vitro*. Henna Rot was tested in the presence and absence of metabolic activation (S9-mix prepared from Aroclor 1254 induced male Sprague-Dawley rat liver). Henna Rot was dissolved in culture medium (MEM). Duplicate cultures of cells were exposed to Henna Rot 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 concentration 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 concentration-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 concentration levels but without a clear concentration-effect relationship.
There was an increase in the number of cells with structural chromosome aberrations (excluding gaps) in both treatment groups with S9-mix at the highest concentration 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 concentration level in any assay.
Positive controls led to increased frequencies of cells with structural aberrations.

Conclusion

Under the experimental conditions used, Henna Rot induced an increase in cells with structural chromosome aberrations and, consequently, is clastogenic and/or aneugenic in human lymphocytes.

Ref.: 19 (subm. I)
27 (subm. II)

In vitro Micronucleus Test in human lymphocytes

Guideline:	OECD 487 (2010)
Cells:	human lymphocytes from 2 healthy, non-smoking male volunteers
Replicates:	duplicate cultures in a single experiment
Test substance:	Henna extract 10%
Batch:	20202207 (content of 2-hydroxy-1,4-naphthoquinone 10%)
Purity:	/
Solvent:	purified water
Concentrations:	10, 20, and 30 µg/ml 3 h treatment without S9-mix 20, 50, and 70 µg/ml 3 h treatment with S9-mix 7.5, 10 and 15 µg/ml 24 h treatment without S9-mix
Treatment:	48 h PHA stimulation, 3 h treatment without and with S9-mix, harvest time 24 h after the start of treatment 48 h PHA stimulation, 24 h treatment without S9-mix, harvest time 24 h after the start of treatment
GLP:	in compliance
Study period:	1 June 2011 – 11 July 2011

Henna extract 10% has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human peripheral blood lymphocytes. The content of the main active ingredient 2-hydroxy-1,4-naphthaquinone with a molecular weight of 174.15 g/mol, also known as Lawsone, was 10% (photometrical purity).
Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.

Test concentrations were based on the results of a cytotoxicity range-finder; cultures of human peripheral blood lymphocytes were treated with a range of 12 increasing concentrations of Henna extract 10% up to 1742 µg/ml, the prescribed maximum concentration of the OECD guideline. The test concentrations were selected by evaluating the effect of Henna extract 10% on the replication index. The highest concentration should produce approximately 60% decrease in replication index.

Both in the cytotoxicity range-finder and the micronucleus test, treatment of lymphocytes commenced approximately 48 h after mitogen stimulation by phytohaemagglutinin. Cells were treated for 3 h both in the absence and presence of S9-mix or 24 h in the absence of S9-mix; cells were harvested 24 h after the beginning of treatment. The final 21-24 h of incubation was in the presence of cytochalasin B (final concentration 6 µg/ml).
Negative and positive controls were in accordance with the draft OECD guideline.

Results

No significant changes in pH or osmolality was observed after the highest concentration tested compared to the untreated controls.
In the absence of metabolic activation, both after 3 h and 24 h treatment a biological relevant increase in the number of lymphocytes with micronuclei was not observed. After 3 h treatment a positive result in isolation was considered not biologically relevant as it was not observed in the duplicate culture. After 24 h exposure, statistical significance was seen after the 2 highest concentrations analysed. A concentration related effect was observed although the number of lymphocytes with micronuclei fell within the normal range of the historical controls. These results could be considered of questionable biological relevance.
In the presence of metabolic activation, treatment with Henna extract 10% resulted in a concentration-dependent and statistically significant increase in the number of cells with micronuclei compared to concurrent solvent controls.

Conclusion

Under the experimental conditions used Henna extract 10% induced an increase in lymphocytes with micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in cultured human peripheral blood lymphocytes.

Ref.: Covance 2011 (submission IV)

In vitro Micronucleus Test in Chinese hamster V79 cells

Guideline:	OECD 487 (2010)
Cells:	Chinese hamster V79 cells
Replicates:	duplicate cultures in a single experiment
Test substance:	Henna extract 10%
Batch:	20202207 (content of 2-hydroxy-1,4-naphthoquinone 10%)
Purity:	/
Solvent:	deionised water
Concentrations:	3.1, 6.3, 12.5, 25.0 and 50 µg/ml without S9-mix 12.5, 25.0, 50.0, 100.0, 200.0 and 400 µg/ml with S9-mix
Treatment:	4 h treatment without and with S9-mix, harvest time 24 h after the start of treatment
GLP:	in compliance
Study period:	13 October 1010 – 3 November 2010

Henna extract 10% has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. The content of the main active ingredient 2-hydroxy-1,4-naphthoquinone with a molecular weight of 174.15 g/mol, also known as Lawsone, was 10.04%. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were

based on the results of a pre-test. V79 cells were treated for 4 h both in the absence and presence of S9-mix and cells were harvested 24 h after the beginning of treatment. Cytotoxicity was measured as a reduction in the proliferation index. The highest concentration should produce approximately 60% decrease in proliferation index. Negative and positive controls were in accordance with the draft OECD guideline.

Results

No significant changes in pH or osmolality were observed after the highest concentration tested compared to the untreated controls. In the absence of metabolic activation precipitation of Henna extract 10% was observed at ≥ 50 µg/ml and in the presence of metabolic activation at ≥ 25 µg/ml.
Both in the absence and presence of metabolic activation, treatment with Henna extract 10% resulted in a concentration-dependent and statistically significant increase in the number of cells with micronuclei compared to concurrent solvent controls in both the precipitating and non-precipitating concentration range.

Conclusion

Under the experimental conditions used Henna extract 10% induced an increase in V79 cells with micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.: Harlan 2010 (submission IV)

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

Guideline:	OECD 479 (1986)
Cells:	Chinese hamster ovary (CHO) cells
Replicates:	duplicate cultures
Test substance:	Henna Rot
Batch:	830.72
Purity:	/
Solvent:	DMSO
Concentrations:	25 – 200 µg/ml without metabolic activation (3 and 24 h exposure) 200 – 800 µg/ml with metabolic activation (3 h exposure)
GLP:	in compliance
Study period:	7 July 1994 – 12 December 1994

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). Henna Rot 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. 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.: 20 (subm. I)
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
Purity:	/
Dose level:	300 mg/kg bw
Route:	i.p.
Vihicle:	1% aqueous carboxymethylcellulose (CMC)
Sacrifice times:	24, 48 and 72 h after treatment
GLP:	in compliance
Study period:	9 January 1992 – 10 February 1992

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 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 Henna Rot 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

Under the experimental conditions used Henna Rot (batch 830.72) did not induce a biologically relevant increase in the number of micronucleated PCEs in bone marrow cells of treated mice and, consequently, Henna Rot is not clastogenic and/or aneugenic in bone marrow cells of mice.

Ref.: 21 (subm. I)
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 (content of 2-hydroxy-1,4-naphthoquinone 1.36%)
Purity:	/
Solvent:	Corn oil
Dose level:	1000 and 2000 mg/kg bw
Route:	oral (gavage)
Sacrifice times:	2 h and 16 h after dosing
GLP:	in compliance
Study period:	22 March 2005 – 31 May 2005

Henna 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 ^{3}H-thymidine. The net nuclear grain counts were determined by counting two slides per animal and 50 cells per slide.
Appropriate positive controls (N,N'-dimethylhydrazine dihydrochloride 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 used, Henna did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 24 (subm. II)

Overview on genotoxicity tests

Batches	**830.72**	**1271**	**20202207**	**Reference**
Preparation	See study	Water 85 °C, extracts prepared by mixing 10 g Henna with 40 ml	Water 37 °C Ultra sound	
Lawsone content	1.2%	1.4%	10%	
***In vitro* gene mutations**				
Gene mutation test in bacteria, study 1	DMSO			5 subm. II

	negative			
Gene mutation test in bacteria	water or DMSO negative			6 subm. II
Gene mutation test in CHO V79 cells, *hprt*-locus	water negative			7 subm. II
Gene mutation test in mouse lymphoma cells, *tk*-locus	Fischer's medium positive			26 subm. II
Gene mutation test in mouse lymphoma cells, *tk*-locus		water negative		23 subm. II
Gene mutation test in mouse lymphoma cells, *tk*-locus		water positive		CIT report 2010, subm. IV
***In vitro* chromosome aberrations**				
Chromosome aberrations test in human lymphocytes	Culture medium (MEM) positive			27 subm. II
Micronucleus test in human lymphocytes			positive	Covance 2011, subm. IV
Micronucleus test in CHO V79 cells			positive	Harlan 2010, subm. IV
Sister Chromatid Exchange test in CHO cells	DMSO negative			8 subm. II
In vivo				
Micronucleus test in mice	1% CMC, i.p. negative			31 subm. II
Unscheduled DNA synthesis test in rats		Corn oil, oral gavage negative		24 subm. II

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

Guideline:	OECD 414 (1981)
Species/strain:	Sprague-Dawley rats Crl CD (SD) BR
Group size:	25 mated females
Test substance:	Henna Rot suspended in 0.5 % aqueous methylcellulose solution
Batch:	830.72
Purity:	/
Dose:	0, 40, 200, 1000 mg/kg bw/d by gavage
GLP:	In compliance
Date:	June 1994 – December 1994

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.

Results
No clinical signs, no abortions and no mortalities were recorded in any female of any group during the study.
A very slight (<10%) but statistically significant decrease of body weight gain and food consumption was observed with the dams receiving 1000 mg/kg/day and was considered by investigators to be 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 soft tissue examination of 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 no ossification 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.

Conclusion
Under the experimental conditions, 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

The study on percutaneous absorption *in vivo* in Sprague Dawles rats was discussed in detail in chapter 3.3.4. In this study also oral administration was investigated in 2 groups of 10 animals (each 5 males and 5 females). The test substance was dissolved in DMSO/water 3:1 and applied by gavage at the doses 37.4 and 39.1 mg/kg bw, respectively. The data show that 96% of the applied substance was excreted within 24 h, 65% was excreted via urine and 35% via feces. The recovery was 96.4%. From this information an oral bioavailability of 65% was deduced and used for the margin of safety calculation. The study results were confirmed by a similar toxicokinetic study with Lawsone (see SCCNFP/0798/04, ref. 48).

Ref.: 9 (subm. II)
Ref.: AR5

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)

Immediate-type allergy to Henna has been reported in the form of presumably IgE-mediated reactions, with symptoms such as sneezing, conjunctivitis, running nose, dry cough, dyspnoea, swelling of the face, or generalized urticaria. The main event for sensitization is inhalation of Henna powder dispersed in the air, but application of pure Henna dough on 1 patient's hand was accompanied by the immediate onset of generalized cutaneous pruritus, rhinitis, conjunctivitis, and decreased expiratory airflow.

Henna-specific IgE has been shown in only one study. One patient with allergic contact dermatitis caused by *p*-phenylenediamine in a Henna tattoo had a positive prick test reaction to 1% Henna in water.

Ref: AR3

3.3.12 Special investigations

A 35 days old boy showed yellowish skin, poor suckling, poor feeding and opisthotonus position for three days. He had jaundice (bilirubin level 50.2 µmol/L), hemoglobinuria and kernicterus symptoms after application of Henna to his skin. In a laboratory test his glucose-6-phosphatase enzyme activity was deficient. There was a history of total bilirubin 12 µmol/L on the 27th day after birth and a history of Henna being applied to the whole body on the 30th day after birth. The author drew the conclusion that this case report

supported the idea that Henna could induce hemolysis in a newborn male with glucose-6-phosphatase deficiency.

Ref.: AR2

SCCS Comment

There is insufficient evidence to draw any conclusion based on the evidence given in this report.

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Lawsonia Inermis (Henna)

The calculation will be based on the default hair dye amount of 35000 mg, with a retention factor of 0.1. Henna contains max. 1.4% Lawsone. A dermal absorption of 4.08% is used. According to SCCNFP/0583/02, the NOAEL of Lawsone is 2 mg/kg bw/d.

SED= E product (expressed in mg/kgbw/d) x C (%) x DA (%)

SED = (35000 mg/ d x 0.1 (Rf) x 1/4(dilution factor) x 1.4/100 x 4.08/100) / 60 kg = 0.00833

SED	**= 0.00833 mg/kg bw/d**
No Observed Adverse Effect Level (NOAEL)	= 2 mg/kg bw/d
Bioavailability 65%	
$NOAEL_{adj}$	**= 1.3**
MOS	**= 156**

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. The batches 1271 and 830.72 of Henna do not vary considerably with regard to the water soluble extract. Since also the Lawsone content is comparable the results of the toxicological studies of these different batches can be used together for the assessment of Henna.

There is an additional batch (20202207) used in 2 genotoxicity tests in submission IV.
Henna Extract 10%, batch number 20202207, was a brown powder. Photometrical content was 10% (content of the main active ingredient Lawsone). Test article stock solutions were prepared by formulating Henna Extract 10% under subdued lighting in purified water, with the aid of vortex mixing, warming at 37 °C and ultrasonication, to give the desired test concentrations.

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.

General 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/day (13 week day rat study); the NOAEL was 200 mg/kg bw/day for the pregnant female rats and 40 mg/kg bw/day for the rat foetuses (teratogenicity study).

Irritation, sensitisation

No separate skin irritation study was performed in experimental animals. However, *Lawsonia inermis* showed no irritant 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 irritant or sensitizing potential under the conditions of RIPT study in Human volunteers.
There are rare case reports of contact allergy to Henna in the literature and rare immediate hypersensitivity reactions.

Percutaneous absorption

In a percutaneous absorption study *in vitro* using pig skin, aqueous Henna slurry 25% was applied for 30 minutes. About 0.28% of the applied dose was found in the receptor fluid and 0.06% remained in the skin.

In a study in which *Lawsonia inermis* pulp was spiked with [^{14}C]-Lawsone the *in vitro* penetration through human skin was:
For the paste A:
2.5 ± 0.3% (1.5 ± 0.22 µg/cm^2 Lawsone applied; 7 chambers, 2 donors) of the applied dose of Lawsone was considered to have penetrated.
For paste B:
5.3 ± 1.0% (1.65 ± 0.01 µg/cm^2 Lawsone applied; 10 chambers, 2 donors)
of the applied dose of Lawsone was considered to have penetrated.

For similar studies with shampoos spiked with [^{14}C]-Lawsone the *in vitro* penetration through human skin was:
For shampoo C:
3.9 ± 0.5% (1.08 ± 0.07 µg/cm^2 Lawsone applied; 24 chambers, 3 donors) of the applied dose of Lawsone was considered to have penetrated.
For shampoo D:
7.2 ± 1.4% (1.00 ± 0.29 µg/cm^2 Lawsone applied; 10 chambers, 2 donors) of the applied dose of Lawsone was considered to have penetrated.

The cutaneous application of the aqueous *Lawsonia inermis* pulp spiked with [^{14}C]-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.

For the three available studies the dermal absorption of Henna (measured by absorption of Lawsone) from a hair dye application the exposure time was not adequate and/or no experimental details were provided. The values varied between 0.2% and 7.2% and 863 ng/cm^2 and 1.7 µg/cm^2, respectively. In comparison, in an *in vitro* study of Lawsone using human skin the absorbed amount of Lawsone is given as 2.6 ± 1.8 µg/cm^2. The SCCS considers that in principle for the MoS calculation the value of paste B (mean + 2SD) should be used adding the amounts in the receptor fluid and in the epidermis and dermis The SCCS

will use a dermal absorption (DAp) of 2.5% + (2 x 0.79) = 4.08% for the calculation of the MoS.

Toxicokinetics

The study on percutaneous absorption *in vivo* in rats was discussed above. In this study also oral administration was investigated. The data show that 96% of the applied substance was excreted within 24 h, 65% was excreted via urine and 35% via feces. The recovery was 96.4%. From this information an oral bioavailability of 65% was deduced and used for the margin of safety calculation.

Mutagenicity

Overall, the genotoxicity of *Lawsonia inermis* (Henna) was investigated for the three endpoints: gene mutations, chromosome aberrations and aneuploidy (see table after Chapter 3.3.6.2.).
Lawsonia inermis batch 830.72 (containing 1.17% Lawsone (2-hydroxy-1,4-naphthoquinone)) did not induce gene mutations in bacteria. In mammalian cells, however, *Lawsonia inermis* batch 830.72 induced an increase in the mutant frequency in the *tk* locus of mouse lymphoma cells but not at the *hprt* locus of V79 cells.
Lawsonia inermis batch 1271 (containing 1.36% 2-hydroxy-1,4-naphthoquinone) induced an increase in the mutant frequency at the *tk* locus of mouse lymphoma cells in one test but was negative in another.
Concerning clastogenicity, *Lawsonia inermis* batch 830.72 induced an increase in the number of human lymphocytes with structural chromosome aberrations. Likewise, *Lawsonia inermis* batch 20202207 induced an increase in the number with micronuclei both in human lymphocytes and in V79 cells. Exposure to *Lawsonia inermis* batch 830.72 did not result in an increase in sister chromatid exchanges.

The positive results found in *in vitro* experiments could not be confirmed in *in vivo* genotoxicity tests. In an *in vivo* micronucleus test using *Lawsonia inermis* batch 830.72 an increase in the number of lymphocytes with micronuclei was not observed. Also an *in vivo* unscheduled DNA synthesis test with *Lawsonia inermis* batch 1271 was negative.
The batches 830.72 and 1271 do not substantially differ from each other concerning the percentage of Lawsone (2-hydroxy-1,4-naphthoquinone; 1.17% *versus* 1.28% respectively) and also with regard to the water soluble extract. The *in vitro* results were not confirmed *in vivo*, on the basis of these tests, these batches can be considered to have no genotoxic potential and additional tests would not be necessary.

It is different with *Lawsonia inermis* batch 20202207 which has a much higher content of the active ingredient 2-hydroxy-1,4-naphthoquinone i.e. 10% and which was clastogenic in a micronucleus test both in human lymphocytes and V79 cells. *Lawsonia inermis* batch 20202207 was not tested in a gene mutation test. To exclude a genotoxic potential of *Lawsonia inermis, batch 20202207* additional testing, *i.e.* a test measuring chromosome aberrations *in vivo* and tests measuring gene mutations *in vitro* and if positive an *in vivo* confirmation test*,* would be required. However, the consumer is not exposed to this kind of extract.

The various studies were performed using different batches as well as different solvents and extraction procedures. The different batches and the test solutions may differ not only with regard to the Lawsone content but also to accompanying constituents of Henna. From the study results it can be derived that Henna's mutagenicity is not only related to the Lawsone content. In order to perform a read across between all the studies a complete characterisation of the test solutions would be required.

Additionally, a reassessment of the genotoxicity of Lawsone by the SCCS is desirable.

4. CONCLUSION

The SCCS is of the opinion that the information provided is sufficient to assess the safe use of Henna as a hair dye. The assessment is based on the Henna batches 1271 and 830.72 and relates to a Lawsone content of max. 1.4%. When formulated and applied as indicated under functions and uses, e.g. 100 g Henna powder mixed with 300 ml boiling water Henna is considered safe for the consumer.

Other kinds of extracts of Henna that may have different compositions are not covered by this assessment.

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

Additionally, a reassessment of the genotoxicity of Lawsone by the SCCS is desirable.

5. MINORITY OPINION

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***Submission IV* (French derogation request)**

Covance Study Number 8248224 Induction of micronuclei in cultured human peripheral blood lymphocytes Report Issued September 2011

Harlan CCR study 1138500 *In vitro* micronucleus test in Chinese Hamster V79 cells with Henna extract 10%, Dec 27, 2010

APPENDIX I
CIT study Henna extract; *in vitro* mammalian cell gene mutation test in L5178Y TK+/" mouse lymphoma cells. Study number 35587 MLY.

APPENDIX II
Henna powder *(Lawsonia inermis* L.): validation of the analytical method for the determination of lawsone in dimethylsulfoxide concentrations: 0.9 and 7 mg/mL and in aqueous henna extract concentrations: 0.9 and 1.4 mg/mL.
Study number 35599 VAA.

APPENDIX III
Henna powder: evaluation of aqueous henna extraction process.
Study number 35943 AHS.

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