Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Red No. 17

(B120)

The SCCS adopted this Opinion at its 10th plenary meeting

on 25 June 2015

Revision of 15 December 2015

About the Scientific Committees

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.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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SCCS

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.).

Scientific Committee members

Ulrike Bernauer, Qasim Chaudhry, Pieter Coenraads, Gisela Degen, Maria Dusinska, Werner Lilienblum, Elsa Nielsen, Thomas Platzek, Christophe Rousselle, Jan van Benthem

Contact
European Commission
Health and Food Safety
Directorate C: Public Health
Unit C2 – Health Information and Scientific Committees
Office: HTC 03/073
L-2920 Luxembourg
SANTE-C2-SCCS@ec.europa.eu

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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SCCS Members

Dr. Qasim Chaudhry
Prof. P.J. Coenraads (chairman)
Prof. M. Dusinska
Dr. W. Lilienblum
Dr. E. Nielsen
Prof. T. Platzek
Dr. S.C. Rastogi (until June 2015)
Dr. C. Rousselle
Dr. J. van Benthem

External experts

Prof. A. Bernard (rapporteur)
Dr. L. Bodin
Prof. J. Duus-Johansen
Dr. J. Ezendam
Prof. A.M. Giménez-Arnau
Dr. E. Mirkova
Dr. E. Panteri
Prof. T. Vanhaecke
Dr. A. Varvaresou

This opinion has been subject to a commenting period of eight weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

Keywords: SCCS, scientific opinion, HC Red No. 17 (B120), Regulation 1223/2009, CAS 1449471-67-3

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1. BACKGROUND

Submission I on the new hair dye with the name HC Red No. 17 (Cosmetics Europe n. B120) CAS No 1449471-67-3 was transmitted by Cosmetics Europe in July 2014.

The new ingredient HC Red No. 17 (B120) is planned to be used in non-oxidative hair colouring products at concentrations of up to 0.5%.

2. TERMS OF REFERENCE

(1) In light of the data provided, does the SCCS consider the new hair dye HC Red No. 17 (B120) safe when used in non-oxidative hair colouring products at concentrations of up to 0.5%?

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Red No. 17 (B120) in cosmetic products?

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

HC Red No. 17 (INCI)

3.1.1.2 Chemical names

Di-[2-[4-[(E)-2-[4-[bis(2-hydroxyethyl)aminophenyl]vinyl]pyridin-1-ium]-butanoyl]aminoethyl]disulfanyl dichloride

CAS Name: Pyridinium, 1,1'-[dithiobis [2,1-ethanediylimino (4-oxo-4,1- butanediyl)]] bis [4- [(1E)-2-[4-] [bis(2-hydroxyethyl) amino] phenyl [ethenyl]-, chloride (1:2)

IUPAC Name: 4-[4-[(E)-2-[4-[bis(2- hydroxyethyl)amino]phenyl]vinyl]pyridin-1-ium-1-yl]-N-[2-[2-[4-[4-[(E)-2-[4-[bis(2hydroxyethyl)amino]phenyl]vinyl]pyridin-1-ium-1-yl] butanoylamino]ethyldisulfanyl]ethyl]butanamide dichloride

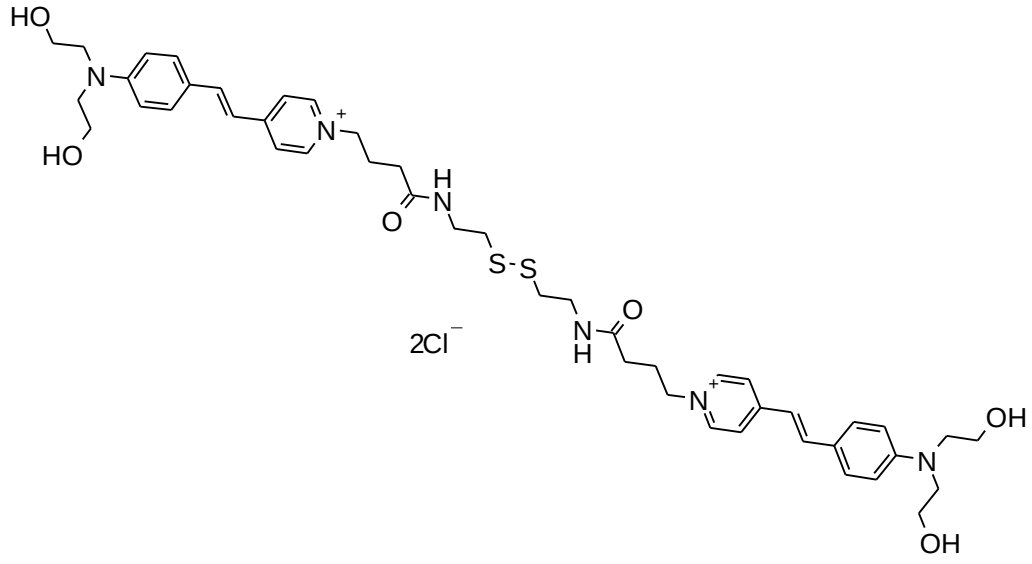
3.1.1.3 Trade names and abbreviations

Vibracolor Garnet Red
Other codes: R0068242A, BCF 52300, Dye B

3.1.1.4 CAS / EC number

CAS: 1449471-67-3
EC: /

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{46}\,H_{62}\,N_6\,O_6\,S_2$, 2Cl

3.1.2 Physical form

Dark red powder

3.1.3 Molecular weight

Molecular weight: 930 g/mol

3.1.4 Purity, composition and substance codes

All studies submitted in the present dossier were conducted using test batch 039 D 001. Chemical characterisation of this batch was performed by elemental analysis and IR, NMR and MS techniques. The purity titre of batch 039 D 001 was 88.5%, and summarised chemical characterisation of this batch is described in the table below. Batch CFQ41584 of [vinyl-2,2'-^{14}C]-R0068242A (97.4% radiochemical purity) was used for the *in vitro* skin absorption study.

Ref. 1, 2

Chemical Characterisation of Vibracolor Garnet batch 039 D 001

Analytical test	batch 039 D 001
HPLC Titre determined against R0068242A 006 L 001 considered as 89.9 % pure*	88.5 % w/w
Impurities (> 0.1%) (Identified at 250 to 700 nm) and content determined against R0068242A006 L 001 considered as 89.9 % pure* (unless otherwise specified)	
Imp. 1	*< 0.1% Area %*
Imp. 2	*0.37 % w/w*
Imp. 3	*2.12 % w/w*
Imp. 4**	*0.14 %* Area %
Imp. 5	0.35 % w/w
Imp. 6	0.39 % w/w
Imp. 7	0.16 % w/w
Imp. 8+9	0.19 % w/w
Imp. 10	0.16 % w/w
Imp. 11	0.13 % w/w
4-[bis(2-hydroxymethyl) amino]benzaldehyde CAS 27913-86-6 content	Not detected (Area %)
Sum of impurities > 0.1%	**3.87 % w/w** 4.01 % (including imp. 4)
Sum of Impurities < 0.1%	0.63 % Area %

* The estimated titre of the reference standard was calculated as: (100 – water content) x UV Relative purity

** Impurity content (UV Spectrum different from UV spectrum of the main product) was evaluated by area %

Chemical Characterisation of Vibracolor Garnet batch 039 D 001(continued)

Analytical test	batch 039 D 001
Water content (% w/w)	5.8
Residual solvents and reagents content (µg/g)	
Pyrrolidine	800
Acetone	<1000
Counter ion (% w/w)	
Chloride (Theoretical: 7.6)	6.2
Sodium content (% w/w)	0.3
Acetate anion	0.35 Mol/mol (NMR)
NaCl content (calculation)	0.8 % w/w
Nitrosamines µg/kg)	
Total N-Nitroso compounds (expressed as NO)	60
Metal content	Nickel <5 ppm; antimony, arsenic, cadmium, lead, mercury: < 1 ppm
Ashes (% w/w)	<0.1
Total %w/w	99.7

3.1.5 Impurities / accompanying contaminants

Potential impurities in Vibracolor Garnet Red originate from starting material, synthesis intermediates, by-products or residual solvents.

The sum of impurities (HPLC, area>0.1%) of batch 039 D 001 of HC Red 17 amounted to 3.87% w/w. Ten molecules, referred to as imp. with numbers 2 to 11, identified by mass spectrometry were fragments /intermediates of HC Red 17.

These impurities included:

- Residual solvents:
  pyrrolidine (800 µg/kg) and traces of acetone (<1000 µg/kg)

- N-nitroso compounds (60 µg/kg)

Concentrations of metals were nickel <5 ppm; antimony, arsenic, cadmium, lead, mercury: < 1 ppm

Chemical composition of two additional batches of HC Red 17 (*not used in the studies presented in the submitted dossier*):

Analytical test	batch Op.4/13	batch CD20/UK, IB3
HPLC Titre determined against R0068242A 006 L 001* considered as 89.9% pure	81.1 % w/w	86.9 % w/w
Impurities (> 0.1%) content determined as area %		
Imp. 1	0.3 % Area % (12.4 min)	0.3 % Area % (10.3 min)
Imp. 2	0.2 % Area % (12.8 min)	0.2 % Area % (19.1 min)
Imp. 3	0.2 % Area % (22.2 min)	0.2 % Area % (29.3 min)
Imp. 4	0.2 % Area % (26.5 min)	2.1 % Area % (31.1 min)
Imp. 5	0.2 % Area % (34.1 min)	0.4 % Area % (37.8 min)
Imp. 6	1.9 % Area % (35.7 min)	0.1 % Area % (41.9 min)
Imp. 7	0.2 % Area % (42.1 min)	0.1 % Area % (44.2 min)
Imp. 8+9	0.4 % Area % (44.3 min)	0.1 % Area % (44.3 min)
Imp. 10	1.1 % Area % (44.5 min)	0.4 % Area % (44.5 min)
Imp. 11	0.3 % Area % (44.7 min)	0.2 % Area % (44.6 min)
		0.1 % Area % (44.7 min)
4-[bis(2-hydroxymethyl) amino]benzaldehyde CAS 27913-86-6	0.29 % w/w	0.38 % w/w)
Sum of impurities > 0.1%	**5.0 % Area %** 5.29 % (including Aldehyde content)	**4.2 % Area %** 4.58 % (including Aldehyde content)
Sum of Impurities < 0.1%	Not determined	Not determined

* The estimated titre of the reference standard was calculated as: (100 – water content) x UV relative purity.

Impurities 1-11 as well as other impurities(<0.01%) have different HPLC retention times in the three batches described .Therefore chemical identity of the impurities in the above two batches cannot be compared with the chemical identity of the impurities of the batch 039 D 001 of HC Red No. 17

Chemical composition of two additional batches of HC Red 17 (continued)

Analytical test	batch Op.4/13	batch CD20/UK, IB3
Water content (% w/w)	3.1	6.2
Residual solvents and reagents content (µg/g)		
Pyrrolidine	5100	200
Ethanol	7,5%	
Counter ion (% w/w)		
Chloride (Theoretical: 7.6)	8.5	10.6
Sodium content (% w/w)	1.16	1.61
Acetate anion	0.9 % w/w	0.1% w/w
NaCl content (calculation)	2.9 % w/w (2)	4.8 % w/w
Nitrosamines µg/kg)		
Total N-Nitroso compounds (expressed as NO)	<50	500*
Heavy metals and ashes		
Ashes (% w/w)	--	--
Metal content	Nickel <5 ppm; antimony, arsenic, cadmium, lead, mercury : < 1 ppm	Nickel <5 ppm; antimony, arsenic, cadmium, lead, mercury : < 1 ppm
Total %w/w	100.7	102.5(2)

* Regarding the high amount of total nitroso content of the sample, the applicant declared that "*it should be emphasised that this sample was purified in the lab. This lab purification step, that we now know, is leading to this high amount of nitrosamines, will not be used any more in the further production process. This could be illustrated by the low nitroso content results shown in the certificate of analysis of the sample Dye B batch Op.4/13 that is representative of the production process*."

3.1.6 Solubility

Solubility was evaluated according to the European Pharmacopoeia protocol, 5.11.
The following values were obtained with batch 039 D 001 at 23 °C.

Ultra Pure Water: 0.1-1g/L
Absolute ethanol: 0.1g/L
DMSO: 10-33g/L
Corn Oil: <0.1g/L

SCCS comment
Water solubility has not been determined according to the EC Method A.6

3.1.7 Partition coefficient (Log P_{ow})

n-Octanol / water partition coefficient measured on Vibracolor Garnet Red batch 033 L 003

Log P_{OW} Calculated: <-2 (ClogPv5.2)
Log P_{OW} Experimental: <-2 (potentiometry)

SCCS comment
Log P_{OW} has not been determined by EC Method A.8

3.1.8 Additional physical and chemical specifications

At room temperature, Vibracolor Garnet Red batch 039 D 001 is a dark red powder.

Melting point:	74°C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	7.7±0.4 (1% w/w at 23±2°C)
UV_Vis spectrum (200-800 nm):	λmax at 270 nm and 469 nm

3.1.9 Homogeneity and Stability

The HPLC analysis of the hair dye formulation showed that the formulation was stable for a 24 hour period (Davies, 2013)

General comments to physico-chemical characterisation
-HC Red 17 is a tertiary amine and thus it is prone to nitrosation. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb.
-Stability of HC Red 17 in typical hair dye formulations has been reported only for a 24 h period
- EC number of HC Red 17 has not been provided.

3.2 Function and uses

HC Red No. 17 is intended to be used in non-oxidative hair colouring products at a maximal concentration of 0.5%.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

No acute oral toxicity studies were performed with HC Red No. 17. However, as no deaths were observed in the oral rat subchronic toxicity study performed at dose levels of up to the limit dose level of 1000 mg/kg/day, it can be inferred that HC Red No. 17 is of low acute toxicity following a single administration by the oral route.

3.3.1.2 Acute dermal toxicity

3.3.1.3 Acute inhalation toxicity

3.3.1.4 Acute intraperitoneal toxicity

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

In Vitro Acute Skin Irritation: Reconstructed Human Epidermis Test Method

Guideline:	*In vitro* Episkin^{SM} Skin Irritation Test, ECVAM validated protocol (ESAC statement 2007)
Test system:	EPISKIN^{SM} Reconstructed Human Epidermis (RHE) Model
Replicates:	3 tissue batches per condition
Test substance:	HC Red No17 (B120)
Test batch:	R0068242A 039 D 001
Purity:	88.5 +/- 0.6%
Test items:	neat (100% powder) and 1% (w/v) raw material in water
Vehicle:	Water in case of the diluted test item
Dose level:	10 mg neat test substance; 10 µl diluted test item
Treatment period:	15 minutes
Post-treatment incubation time:	42 hours
Positive control:	5% (w/v) aqueous solution of sodium dodecyl sulphate (SDS)
Negative control:	PBS+
Solvent control:	Sterile water
Direct interaction with MTT:	Negative
Colouring of epidermis:	Positive
GLP:	In compliance
Study period:	April 2012

Nine tissues per condition were treated with neat and 1% diluted test item for an exposure period of 15 minutes. The test item in undiluted and diluted form was applied topically to the corresponding tissues ensuring uniform covering. Approximately 10 mg of neat test substance and 10 µl diluted test item were applied to the epidermis surface. The epidermis surface devoted for neat substance testing had previously been moistened with 5 µl of distilled water to improve contact between the solid test item and the epidermis. Nine tissues treated with 10 µl PBS+ served as the negative controls, nine tissues treated with 10 µl SDS 5% w/v served as the positive controls and nine tissues treated with 10 µl distilled water served as the solvent controls for the 1% test item. In order to allow correction for staining of the tissue by the colouring agent, three additional tissues followed the same treatment as the other tissues except for the MTT incubation period. The plates were kept at room temperature for 15 minutes ± 1 minute. At the end of the exposure period, each tissue was rinsed with PBS+. The rinsed tissues were transferred to the appropriate wells of a 12-well plate previously prepared with 2 ml of maintenance medium. The rinsed tissues were incubated at 37°C, 5% CO_2 for 42±1 hours. The negative and positive control groups were rinsed using the same process. Following the 42-hour post-treatment incubation period, three tissues for each treatment group were transferred to 12-well plates containing 2 ml of 0.3 mg/ml MTT solution, freshly prepared in assay medium. The tissues were incubated for 3 hours at 37°C, 5% CO_2 and 95% humidity. At the end of the 3-hour incubation period, the tissues were examined and the degree of MTT staining was evaluated. The maintenance culture media were kept frozen for further cytokine IL-1α measurements. Following qualitative evaluation of tissue viability, a total biopsy of the epidermis was made. The epidermis was carefully separated from the collagen matrix and both parts placed into micro tubes containing 500 µl of acidified isopropanol. The extraction of formazan crystals was performed in protection of light at 5 °C. At the end of the formazan extraction, the optical density was measured at 570 nm versus acidified isopropanol as blank and the % cell viability was calculated. Additionally, the level of IL-1α released in the culture medium was determined by a classic sandwich enzyme immunoassay technique (ELISA). 200 µl of standards of culture medium samples were added to the wells enabling IL-1α to bind to immobilised antibody. After multiple washings and developing steps, a colour develops in proportion to the amount of IL-1α bound. The intensity of the resulted colour (OD values related to the IL-1α amount) was measured at 450 nm.

Results

HC Red No17 did not induce a significant decrease in cell viability in the MTT assay. The mean viability value for neat HC Red No 17 and HC Red No 17 diluted at 1% (w/v) was 97.9% and 96.5%, respectively. The standard deviations of the viability (2.2 and 5.3, respectively) were within the acceptance range (≤ 18%). The negative, positive and vehicle controls confirmed the validity of the assay. The final IL-1α release for neat and 1% diluted test substance was 11.1 pg/ml and 4.6 pg/ml, respectively.

Conclusion

Under the conditions of the study, the *in vitro* evaluation of acute skin irritation by using the reconstructed human epidermis Episkin^{SM} model, suggests that HC Red No 17 undiluted and diluted at 1% in water as non-irritant (MTT viability > 50%, IL-1α release <50 pg/ml).

Ref. 1, 2

3.3.2.2 Mucous membrane irritation / Eye irritation

Bovine Corneal Opacity and Permeability (BCOP) Test Method

Guideline:	OECD 437 (September 2009)
Test system:	Isolated bovine eyes (corneas)
Group size:	6 corneas per condition

Test substance:	HC Red 17 (B120)
Batch:	R0068242A 039 D 001
Purity:	88.5 ± 0.6%
Test item:	20 % (w/w) in physiological saline
Vehicle:	Physiological saline (NaCl 0.9%)
Dose level:	750 µl
Treatment period:	4 hours
Positive control:	20% (w/w) imidazole in NaCl 0.9%
Negative control:	NaCl 0.9% in sterile water
GLP:	In compliance
Study period:	May - June 2013

Bovine eyes (from cattle less than 12 months old) were collected at slaughterhouses and prepared within 4 hours of collection. Eyes that were too big or were presenting defects were rejected. After the pre-incubation and equilibration period of the corneas of at least 1 hour at 32 ± 1°C, the test item was applied onto the corneas. The treatment period of 4 hours ± 10 minutes was followed by a rinsing step and visual examination of rinsing efficiency. The treatment procedure was the same for both the test item and the control-treated tissues. Then, 6 corneas per concentration were used to measure the corneal opacity. 3 other corneas were incubated with 0.5% fluorescein solution for 90 ± 5 minutes at 32 ± 1°C and used for measurement of the corneal permeability by measuring optical density at 490 nm. Based on the obtained mean values of opacity and permeability, the *in vitro* irritancy score (IVIS) was calculated. The remaining 3 corneas were kept for histological analysis. For each sample, 5 sagittal sections per cornea were taken (centre, the whole length of the cornea, along its taller diameter) in order to observe any possible lesions induced by the test item. The tissues were examined and the findings were grated using a five point system of minimal (grade 1), slight (grade 2), moderate (grade 3), marked (grade 4) or severe (grade 5). Ocular effects were evaluated using the endpoints of the corneal opacity and permeability as well the histological analysis of the treatment tissues.

Results

HC Red No 17 tested at 20% (w/w) in NaCl 0.9% does not have a significant effect on the corneal opacity and permeability. The IVIS value (6.5±1.7) obtained after 4-hour contact with the cornea was in the acceptance range for it not to be classified as corrosive or as a severe irritant (IVIS < 55.1). The results for positive and negative controls confirmed the validity of the study. The corrected opacity value (126.3) for 20% (w/w) imidazole solution in NaCl 0.9% was within the acceptance interval (120-200). The optical density value for negative control corneas (0.058) was within the accepted tolerance range (< 0.1). The histological analysis of tissues treated with 20% (w/w) HC Red No 17 showed no evidence of topical irritation versus negative controls. Application of the positive control induced changes in the epithelium characterised by minimal to slight erosion / ulceration, moderate cell dissociation and cell alteration as well as minimal to marked epithelium / stroma dissociation.

Conclusion

From the results obtained under the conditions of this study, the test item HC Red No 17 at 20 % (w/w) in 0.9% NaCl is not classified as corrosive or as a severe irritant for the isolated bovine cornea, after 4 hours of exposure. The application of 20% HC Red No 17 on the cornea did not induce any histological evidences of topical irritation.

SCCS comment

On the basis of the results obtained in the BCOP study, it can be concluded that HC Red No 17 diluted to 20% (w/w) in 0.9% NaCl is not a strong eye irritant. This, however, does not exclude a mild or moderate eye irritancy potential. Under the conditions of this study, an eye irritation potential of HC Red No 17 at 20% (w/w) cannot be excluded. Considering that

the maximum intended concentration of HC Red No 17 in a hair dye product is 0.5% (w/w) and no severe eye irritation potential is observed in the BCOP test for 20% (w/w) HC Red No 17, it can be assumed that eye irritation will be of limited concern.

Ref. 3

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species/strain:	mice/CBA/J
Group size:	4
Test substance:	HC Red 17 (B120)
Batch:	039 D 001
Purity:	88.5 +/- 0.6% pure
Vehicle:	DMSO
Concentration:	0, 1, 2.5, 5, 10, 25% (w/v)
Positive control:	alpha-hexylcinnamaldehyde (HCA)
GLP:	in compliance
Study period:	December 2011 - January 2012

Test procedure

Twenty-eight (28) female CBA/J mice were used in the main study in order to assess the skin sensitisation potential of HC Red No. 17.
Animals were separated in 7 groups (4 mice/group) consisting of:

- 5 treated groups receiving HC Red No. 17 at 1, 2.5, 5, 10 or 25% (w/v) in DMSO.
- Due to unsatisfactory solubility tests of HC Red No. 17 in the first recommended vehicles, DMSO was selected. A solubility study showed that 50% (w/v) HC Red No. 17 in DMSO was the maximal achievable concentration.
- The pre-test showed that treatment with 50% HC Red No. 17 caused a 40% increase in ear thickness, and this concentration therefore was not selected for the main study.
- A negative control group receiving the vehicle (DMSO) alone
- A positive control group receiving alpha-hexylcinnamaldehyde (HCA) at 25% (v/v) in DMSO

The test substance HC Red No. 17, DMSO or HCA was applied on the dorsum of the ears (25 µL per ear) of the animals for three consecutive days designated as days 0, 1 and 2. After 2 days of resting (day 5), mice received a single intravenous injection of tritiated methyl thymidine (^{3}H-TdR). Lymph nodes draining the application sites (auricular nodes) were sampled, pooled per group, and the proliferation of lymphocytes was evaluated by measuring the incorporation of ^{3}H-TdR. The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed by measuring ear thickness on days 0, 2 and 5.

Results
The validity criteria were fulfilled. In particular, a SI value of 5.21 was obtained with the positive control HCA. SI values of 1.42, 1.53, 1.75, 2.36 and 2.55 were obtained at HC Red No. 17 concentrations of 1%, 2.5%, 5%, 10% and 25%, respectively.

Conclusion
Under the conditions of this study, HC Red No. 17 did not induce delayed contact hypersensitivity and was therefore considered to be devoid of sensitising potential.

Ref. 4

SCCS comment
HC Red No. 17 induced a dose-dependent increase of lymphocyte proliferation in the LLNA that resulted in a SI value of 2.55 at the highest dose (25%) tested. HC Red No. 17 is not considered to be a skin sensitiser under the conditions of the test.

3.3.4 Dermal / percutaneous absorption

Guideline:	OECD 428 (November 2004); SCCS 1358/10
Species/strain:	Frozen human dermatomed skin
Membrane integrity:	Assessed by measurement of electric resistance across the membrane
Group size:	12 intact skin membranes from 4 different female donors
Method:	Human dermatomed skin
Test substance:	HC Red 17 (B120)
Batch:	R0068242A 039 D 001 CFQ41584 of [vinyl–2,2'-^{14}C]-R0068242A
Purity:	Chemical 88.5 ± 0.6% Radiochemical (HPLC) 98.8%
Test item:	5,84mg R0068242A/g labelled with vinyl–2,2'-^{14}C
Exposed membrane area:	2.54cm^2
Dose applied:	20 mg/cm² corresponding to a nominal dose rate of 117 µg active ingredient /cm²
Sample volume:	0.5ml
Sampling period:	24 hours
Receptor fluid:	Phosphate buffered saline - degassed
Mass balance analysis:	Provided
Tape stripping:	Yes (20)
Method of Analysis:	Liquid Scintillation Counting (LSC)
GLP:	In compliance
Study period:	March - October 2013

Twelve human back and abdominal skin samples were obtained from four different female donors from a tissue bank. The membranes were stored frozen, at approximately -20° C, on aluminium foil until use. Skin samples were dermatomed (400 µm in thickness). Discs of approximately 3.3 cm diameter of prepared skin membrane were mounted, dermal side down, in diffusion cells held together with individually numbered clamps and placed in a water bath maintained at 32°C ± 1°C. Prior to dosing, the membrane integrity was checked by measurement of the electrical resistance across the skin membrane. Membranes with a measured resistance of <10 kΩ were regarded as having a lower integrity than normal and rejected. The receptor chambers of the cells containing small magnetic stirrer bars were filled with a recorded volume of degassed phosphate buffered saline (PBS) receptor fluid. Following preliminary investigations, HC Red No. 17 was shown to bind to the glassware, which was therefore treated with 5% dimethyldichlorosilane in toluene. This treatment yielded a good recovery rate. A typical hair dye formulation containing about 0.5% [^{14}C]-HC Red No. 17 was tested. 20 mg/cm² of this formulation (corresponding to a nominal dose rate of 117µg/cm² of HC Red No. 17) was applied to the skin surface and left for 20 minutes to mimic in-use conditions. A sample of the receptor fluid (0.5 mL) was taken manually 20 minutes after application after which the samples (0.5 mL) were taken using an autosampler at 1, 2, 4, 8, 12, and 24 hours after application for analysis by LSC. At the end of the 20-minute exposure period, the remaining formulation on the skin surface was removed using a standardised washing procedure, simulating use conditions. Twenty-four (24) hours after application, the percutaneous absorption of HC Red No. 17 was estimated by measuring its concentration by liquid scintillation counting in the following compartments: skin washes, *stratum corneum* (isolated by 20 tape strips), living epidermis/dermis, unexposed skin and receptor fluid.

Results
All diffusion cells yielded data that could be analysed and the mean recovery rate was good at 95.6%. The HPLC analysis of the hair dye formulation performed following the dosing procedure and 24 hours post application was 98.8 and 98.6%, respectively, confirming that the formulation was stable for a 24-hour period. Most of the HC Red No. 17 applied on the skin surface was removed with the skin wash at 20 minutes (95.5%). The mean amount of HC Red No. 17 considered as systemically available was estimated as the sum of the amounts measured in the remaining skin after tape stripping, the dermis and the receptor fluid: 0.011 ± 0.015 µg-eq/cm^2 (0.01 ± 0.013% of the applied dose).

	µg-eq/cm² of HC Red No. 17		% of applied dose	
	Mean	SD	Mean	SD
Donor chamber	0.007	0.005	0.006	0.004
Skin wash at 20 minutes	112	2.73	95.5	2.34
Skin wash at 24 hours	0.143	0.136	0.122	0.117
Stratum corneum	0.009	0.009	0.008	0.008
Remaining skin	0.010	0.015	0.009	0.013
Dermis	0.0006	0.0005	0.0005	0.0005
Flange	0.005	0.005	0.005	0.005
Receptor fluid	0.0003	0.0002	0.0002	0.0002
Total non-absorbed	112	2.72	95.6	2.33
Systemically available	0.011	0.015	0.010	0.013
Total recovered	112	2.72	95.6	2.33

Total non-absorbed = Sum of donor chamber, skin wash, flange and *Stratum corneum*
Systemically available = Sum of remaining skin, dermis and receptor fluid
Skin wash at 20 minutes = Sum of 20 minute sponge, 20 minute pipette and 20 minute skin wash
Stratum corneum = Amount in tape strips. Remaining skin = Tissue remaining after tape stripping

Conclusion
The results obtained in this study indicate that HC Red No. 17 present at 0.5% in a typical hair dye formulation penetrates through human dermatomed skin at an extremely slow rate. The amount of HC Red No. 17 considered as absorbed was estimated to be at most 0.011 ± 0.015 µg-eq/cm^2 corresponding to 0.01 ± 0.013% of the applied dose.

Ref. 5

SCCS comment
Exposure time in the study was unusually short i.e. 20 instead of the recommended 30 minutes for hair dyes. Thus, the dermal absorption may be underestimated. Therefore the mean +2 standard deviations will be used for the MoS calculation: 0.011 + 2 x 0.015 = 0.041 µg-eq/cm^2.

3.3.5 Repeated dose toxicity

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

Guideline: OECD 408
Species/strain: Rat/Wistar
Group size: 10 females and 10 males

Test substance:	HC Red 17 (B120)
Batch:	Test batch 039 D 001
Purity:	88.5 +/- 0.6%
Vehicle:	1% (w/v) aqueous methylcellulose and 1% (v/v) Tween® 80
Dose levels:	0, 100, 300, 1000 mg/kg bw/day
Dose volume:	10 ml/kg
Route:	oral
Administration:	gavage
GLP:	in compliance
Study period:	July 1 to October 4, 2012

Test procedure

The subchronic toxicity of HC Red No. 17 was investigated in Wistar rats (10/sex/group) after daily oral gavage at 0, 100, 300 or 1000 mg/kg bw in 1% (w/v) aqueous methylcellulose and 1% (v/v) Tween® 80 (10 ml/kg bw) for 13 weeks. These dose levels were selected on the basis of the results of a preliminary 14-day study performed at 100, 300 and 1000 mg/kg/day where no mortality, clinical signs, or important changes in bodyweight, bodyweight gain or food consumption were observed. No toxicological changes were observed in haematological, coagulation, clinical chemistry, or urinalysis parameters, or upon macroscopic or microscopic examination of tissues/organs. Evaluations and measurements included mortality checks, daily clinical observations, weekly bodyweight and food intake, ophthalmoscopy prior to dosing and at the end of the treatment period, neurotoxicological evaluation during week 12, haematology, blood clinical chemistry and urinalysis (week 13). At the end of treatment period, surviving animals were killed and subjected to macroscopic examination; selected organs were weighed, and a wide range of organs/tissues was preserved. Microscopic examination was performed for specified tissues/organs from control and high dose rats killed at the end of the dosing period, as well as for any gross anomaly.

Results

The chemical analysis of the dose formulations administered during the study showed that achieved concentrations were close to the intended values. The test item did not induce any relevant treatment-related changes with respect to survival, clinical signs, ophthalmological examinations as well as haematology, coagulation, urine and clinical chemistry parameters evaluated. No test item-related changes were detected regarding absolute and relative organ weights, gross and microscopic examination. No toxicologically significant test item-related neurological abnormalities were observed. Pinkish urines were observed at 300 and 1000 mg/kg dose levels and were related to the renal elimination of coloured test item material and were thus considered to be evidence of systemic exposure of the animals following oral administration of HC Red No. 17. Reddish brown coloured faeces were observed during the treatment period in all dose groups but were due to the colour of the test item. Moreover, reddish gastrointestinal tract contents were observed in all dose groups. These effects due to the coloured test item were considered as non-adverse as they were not associated with gross or microscopic changes in the mucosa of the gastro-intestinal tract. There were no consistent changes in body weights and food consumption at the dose levels of 100 and 300 mg/kg/day in males and at all dose levels in females. When compared to controls, significant lower food consumption associated with a decrease in mean bodyweights and bodyweight gains were observed at the dose of 1000 mg/kg/day in males.

Conclusion

The daily oral administration for 13 weeks of HC Red No. 17 to rats at dose levels of 100, 300 or 1000 mg/kg bw was well tolerated. Thus, under the conditions of the study, the NOAEL (No Observed Adverse Effect Level) of this 90-day oral toxicity study on HC Red No. 17 was 300 mg/kg bw/day in Wistar rats.

Ref. 6

SCCS comment
The feed consumption was consistently decreased only in males at the highest dose of HC Red No. 17. Sporadically, there were statistically significant variations of feed consumption at lower doses. However, these variations were inconsistent and therefore unlikely to be treatment-related. A two-way ANOVA testing both the effects of dose and time would have been a more appropriate test than the one-way ANOVA test used in the study.

3.3.5.2 Chronic (> 12 months) toxicity

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Test system:	*Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102
Replicates:	2 experiments
Test substance:	HC Red 17 (B120)
Batch:	R0068242A 039 D 001
Purity:	88.5 ± 0.6 %
Concentrations:	Experiment I: with and without S9-mix, 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate Experiment II: with and without S9-mix, 20.48, 51.2, 128, 320, 800, 2000, 5000 µg/plate
Treatment:	Experiment I: direct plate incorporation, with and without S9-mix Experiment II: direct plate incorporation without S9-mix and pre-incubation with S9-mix
Vehicle:	purified water
GLP:	In compliance
Study period:	October 25 to November 13, 2011

Results
The negative control counts fell within the normal ranges. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9-mix preparation. Following these treatments, evidence of toxicity in the form of a slight thinning of the background bacterial lawn or a complete killing of the test bacteria was observed at 1000 and/or 5000 µg/plate in all strains in the absence and presence of S9-mix. Following R0068242A treatments of all the test strains in the absence and presence of S9-mix, no increases in revertant numbers were observed that were statistically significant when the data were analysed at the 1% level using Dunnett's test. This study was considered therefore to have provided no evidence of any R0068242A mutagenic activity in this assay system.

Conclusion
Under conditions of the experiments, HC Red No. 17 was not mutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102, either in the presence or absence of metabolic activation.

Ref. 7

Mammalian Cell Gene Mutation Test in Mouse Lymphoma Cells (*hprt* locus)

Guideline:	OECD 476
Test system:	Mouse lymphoma cell line L5178Y [*hprt* locus]
Replicates:	duplicate
Test substance:	HC Red 17 (B120)
Batch:	batch 039 D 001
Purity:	88.5 +/- 0.6% pure
Concentrations:	Experiment I: without S9-mix: 100, 200, 800, 1000, 1100, 1200, 1300 and 1500 µg/mL with S9-mix: 50, 100, 150, 200, 300, 400, 500 and 600 µg/mL Experiment II: without S9-mix: 400, 800, 1200 and 1300 µg/mL with S9-mix: 50, 100, 200, 300 and 400 µg/mL
Treatment:	Experiment I and II: 3 hour treatment without and with S9-mix; an expression period of 7 days and a selection period of 10-15 days
Vehicle:	purified water
GLP:	In compliance
Study period:	20 October 2011 – 28 December 2011

The mouse lymphoma cell line L5178Y [*hprt* locus for 6-thioguanine resistance] was used.

The test item HC Red No. 17 was evaluated in two independent experiments using duplicate cultures each (single cultures for positive controls). Both experiments used a pulse (3-hour) treatment and were conducted in the absence and presence of metabolic activation (S9-mix prepared from the liver of rats given Aroclor 1254). The ingredient HC Red No. 17 was tested in both experiments at a broad range of concentrations. The concentrations were selected on the basis of both cytotoxicity and solubility criteria. Known mutagens in the presence (Benzo(a)pyrene, BP) or absence of S9-mix (4-nitroquinoline 1-oxide, NQO) were tested at two different concentrations and served as positive controls. Negative controls consisted of cultures treated with the solvent alone (purified water).

Results

The appropriate levels of toxicity (10-20% relative survival after the highest concentration) were reached in the absence of S9-mix but not in the presence of S9-mix.
Mutant frequencies in solvent negative controls fell within normal ranges, and treatment with positive controls NQO and BP yielded distinct increases in mutant frequency. Accordingly, the study was considered to be valid. No biologically relevant increase in mutant frequency was observed in either experiment following treatment with HC Red No. 17 at any concentration tested in the absence and presence of metabolic activation.

Conclusion

Under the conditions of this study, HC Red No. 17 was considered not to be mutagenic in the mouse lymphoma assay (*hprt* locus), either in the absence or presence of metabolic activation.

Ref. 8

SCCS comment

The required level of toxicity (10-20% relative survival after the highest concentration) was not reached in the presence of S9-mix.

***In Vitro* Micronucleus Test in Cultured Human Lymphocytes**

Guideline:	OECD 487
Test system:	*In vitro* micronucleus test in human lymphocytes
Replicates:	duplicate (four replicates for vehicle)
Test substance:	HC Red 17 (B120)
Batch:	batch 039 D 001
Purity:	88.5 +/- 0.6% pure
Concentrations:	Experiment I: without S9-mix 250, 300 and 350 µg/mL Experiment II: with S9-mix 150, 200 and 250 µg/mL Experiment III: without S9-mix 200, 250 and 350 µg/mL
Treatment:	Experiment I: 3 h treatment Experiment II: 3 h treatment Experiment III: 24 h treatment
Vehicle:	purified water
GLP:	In compliance
Study period:	October 20-December 28, 2011

HC Red 17 was assayed for the induction of micronuclei in cultured human peripheral blood lymphocytes from two male volunteers in the absence and presence of metabolic activation (S9 mix prepared from the livers of Aroclor 1254-treated rats). The highest concentration in each test condition was selected on the basis of solubility criteria since no cytotoxicity was observed with the test item. Top concentrations were then selected based on precipitation of the test item observed at harvest.

Duplicate cultures were treated with each concentration of HC Red No. 17 or with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9-mix (mitomycin C, MMC and vinblastine, VIN). Solvent-treated cultures (purified water, four replicates) were used as negative controls. Blood cultures were incubated in the presence of the mitogen phytohaemaglutinin (PHA) for 48 hours and then treated for 24 or 3 hours in the absence or presence of S9-mix, respectively. Cells were harvested 72 hours after the beginning of incubation. Cytochalasin B was added after the 3-hour treatments or before the 24-hour treatments. Lymphocyte preparations were stained and examined microscopically for determining the replication index (RI) and the proportion of micronucleated binucleated (MNBN) cells when selected. Two thousand binucleate cells per concentration (one thousand from each replicate) were analysed blindly.

Results

When compared to concurrent solvent controls, treatment of cultures with positive controls CPA, MMC and VIN resulted in consistent significant increases in MNBN frequencies, thus validating the sensitivity of the test system and procedure used. Treatment of cells with HC Red No. 17 in the absence and presence of S9-mix resulted in frequencies of MNBN cells, which were similar to those observed in concurrent vehicle controls for all concentrations analysed under all treatment conditions.

Conclusion

Under the conditions of the study, HC Red No. 17 did not produce micronuclei in cultured human peripheral blood lymphocytes either in the absence or presence of metabolic activation and was therefore considered to have no clastogenic or aneugenic potential.

Ref. 9

SCCS comment

Genotoxicity tests of HC Red No. 17 (B120) were performed by dissolving the compound in purified water. Because of the low solubility of HC Red No. 17, test concentrations were limited by precipitation and not by cytotoxicity. The solubility of HC Red No. 17 could have been maximised by using an organic solvent such as dimethyl sulfoxide (DMSO). This, however, would not have changed the risk evaluation as the overall experimental evidence

clearly shows that HC Red No. 17 is a compound with a very low toxicity, which is only negligibly absorbed through the skin.

3.3.6.2 Mutagenicity / Genotoxicity in vivo

3.3.7 Carcinogenicity

No data submitted.

3.3.8 Reproductive toxicity

3.3.8.1 Two-generation reproduction toxicity

3.3.8.2 Other data on fertility and reproduction toxicity

3.3.8.3 Developmental Toxicity

Guideline:	OECD 414
Species/strain:	Rat/Wistar
Group size:	24
Test substance:	HC Red 17 (B120)
Batch:	039 D 001
Purity:	88.5 +/- 0.6%
Vehicle:	methylcellulose and 1% (v/v) Tween® 80
Dose levels:	0, 100, 300 or 1000 mg/kg bw/day
Dose volume:	10 mL/kg
Route:	oral
Administration:	gavage
GLP:	in compliance
Study period:	July 1 to October 4, 2012

Test procedure
The potential effects of HC Red No. 17 on pregnant rats and embryo-foetal development were evaluated through daily oral gavage in which mated Wistar female rats (24/group) were dosed at 0, 100, 300 or 1000 mg/kg/day during the sensitive period of organogenesis from gestation day 5 to day 19 [the day of mating was designated as Gestation Day 0 (GD 0)]. The test item was dissolved in 1% (w/v) aqueous methylcellulose and 1% (v/v) Tween® 80 and given at 10 mL/kg. These dose levels were selected on the basis of the results of a preliminary study performed at 100, 300 and 1000 mg/kg/day where no adverse effects were observed [Ravi, 2013]. Maternal evaluations and measurements included daily clinical signs and bodyweight/food intake measured at designated intervals. The dams were killed on GD 20 and subjected to macroscopic examination. Usual litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies, and remaining foetuses were examined for skeletal anomalies.

Results
The chemical analysis of the dose formulations administered during the study showed that achieved concentrations were close to the intended values. The administration of HC Red No. 17 to pregnant female Wistar rats over the organogenesis period produced no mortality or gross necropsy findings. Reddish brown faeces were observed from GD 6 to GD 20, which

could be attributed to the red brown colour of the test item and hence were considered to be non-adverse. Maternal toxicity occurred only at the highest dose level of 1000 mg/kg/day such as lower mean body weight, body weight gain, corrected bodyweight gain and food intake when compared to controls. No changes in maternal and litter parameters were noted in any group. There were no foetal external, visceral and skeletal findings attributed to the administration of the test item.

Conclusion
On the basis of the results obtained in the present study, and specifically the effects on maternal bodyweight, the No Observed Adverse Effect Levels (NOAEL) for maternal and developmental toxicity of HC Red No. 17 were respectively set at 300 mg/kg/day and 1000 mg/kg/day. HC Red No. 17 was considered to have no teratogenic potential.

Ref. 10

3.3.9 Toxicokinetics

3.3.9.1 Toxicokinetics in laboratory animals

3.3.9.2 Toxicokinetics in humans

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

3.3.10.2 Photomutagenicity / photoclastogenicity

3.3.11 Human data

3.3.12 Special investigations

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(20mg/cm^2 applied to the skin for 20 minutes)
(0.5 % formulation, on-head concentration 100 µg/cm^2)

Absorption through the skin	A	=	0.041 µg/cm²
Skin Area surface	SAS	=	580 cm^2
Dermal absorption per treatment	SAS x A x 0.001	=	0.024
Typical body weight of human		=	60 kg
Systemic exposure dose (SED)	SAS x A x 0.001/…	=	0.00040 mg/kg bw
No observed adverse effect level (90-day, oral, rat)	NOAEL	=	300 mg/kg bw/d
Bioavailability 10%*		=	30 mg/kg bw/d

Margin of Safety	**adjusted NOAEL/SED = 75.000**

* Because of the large molecular weight and the ionic character of the dye, 10% bioavailability is considered.

3.3.14 Discussion

Physico-chemical properties

HC Red No. 17 or Vibracolor Garnet Red is a dark red powder. The product is very slightly soluble in water and practically insoluble in corn oil and ethanol.

HC Red No17 is intended to be used as a non-oxidative hair colouring product at concentrations up to 0.5%

HC Red 17 is a tertiary amine and thus it is prone to nitrosation. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb. Stability of HC Red 17 in typical hair dye formulations has been reported only for a 24-h period.

General toxicity

No acute oral toxicity studies were performed with HC Red No. 17. However, as no deaths were observed in the oral rat subchronic toxicity study performed at dose levels of up to the limit dose level of 1000 mg/kg/day, it can be inferred that HC Red No. 17 is of low acute toxicity following a single administration by the oral route.

The subchronic toxicity of HC Red No. 17 was investigated in a 13-week oral toxicity study where rats were given HC Red No. 17 by gavage at 100, 300 or 1000 mg/kg/day. There were no adverse effects, with only signs related to the staining properties of HC Red No. 17 (pinkish urine and reddish brown faeces) observed at 300 and 1000 mg/kg/day. Coloured urine was indicative of renal excretion of the test material, thus showing systemic exposure of the animals following oral administration of HC Red No. 17. When compared to controls, significantly lower food consumption associated with lower mean bodyweights and bodyweight gains were observed at 1000 mg/kg/day in male rats only. Accordingly, the No Observed Adverse Effect Level (NOAEL) for this study was considered to be 300 mg/kg/day.

The potential effects of HC Red No. 17 on embryo-foetal development were evaluated in a study performed by the oral route (gavage) in which pregnant rats were given HC Red No. 17 during the sensitive period of organogenesis. In this study performed at 100, 300 or 1000 mg/kg/day, maternal toxicity was observed at the highest dose level and consisted of lower mean bodyweight, bodyweight gain, corrected bodyweight gain and food intake when compared to controls. There were no foetal findings attributed to the administration of the test material. Accordingly, under the conditions of this study, the NOAELs for maternal and embryo-foetal toxicity were set at 300 and 1000 mg/kg/day, respectively. Additionally, HC Red No. 17 was considered to have no teratogenic potential.

Irritation/sensitization

The results obtained in *in vitro* skin irritation tests on Human Reconstructed Epidermis (Episkin^{SM}) suggested that HC Red No. 17 has no skin irritation potential when tested either neat or diluted at 1%. Similarly, the results obtained in an acute ocular irritation *in vitro* test i.e. the Bovine Corneal Opacity and Permeability (BCOP) test suggested that a 20% dilution of HC Red No. 17 has no eye irritation potential. Considering that the maximum intended concentration of HC Red No 17 in a hair dye product is 0.5% (w/w) and no severe eye irritation potential is observed in the BCOP test for 20% (w/w) HC Red No 17, it can be assumed that eye irritation will be of limited concern.

The skin sensitising potential of HC Red No. 17 was evaluated in a murine Local Lymph Node Assay (LLNA) conducted at concentrations up to the maximum non-irritating concentration of 25%. A dose-dependent increase of lymphocyte proliferation in the LLNA was induced by HC Red No. 17, but the highest concentration tested induced a SI value of 2.55. Therefore, HC Red No. 17 was not considered to be a skin sensitizer under the conditions of the test.

Dermal absorption

The *in vitro* percutaneous absorption of HC Red No. 17 was determined in human dermatomed skin using a typical non-oxidative hair colouring formulation containing [14C]-HC Red No. 17 at 0.5%. The formulation was applied under use conditions. After 24 hours, the absorbed amounts (sum of amounts contained in living epidermis/dermis and receptor fluid) were estimated to be at most 0.011 ± 0.015 µg-eq/cm2 (0.01 ± 0.013% of the applied dose).

Exposure time in the study was unusually short. Therefore the mean +2 standard deviations will be used for MoS calculation: 0.011 + 2 X 0.015 = 0.041 µg-eq/cm^2.

Mutagenicity

HC Red No. 17 was investigated in genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, structural and numerical chromosome aberrations. HC Red No. 17 did not induce gene mutations in bacteria nor in mammalian cells when evaluated at the *hprt* locus. Exposure of human lymphocytes with HC Red No. 17 did not result in an increase of micronucleated binucleated cells.

Genotoxicity tests of HC Red No. 17 (B120) were performed by dissolving the compound in purified water. Because of the low solubility of HC Red No. 17, test concentrations were limited by precipitation and not by cytotoxicity. The solubility of HC Red No. 17 could have been maximised by using an organic solvent such as dimethyl sulfoxide (DMSO). This, however, would have not changed the risk evaluation as the overall experimental evidence clearly shows that HC Red No. 17 is a compound with a very low toxicity, which is only negligibly absorbed through the skin.

Based on the present available tests, HC Red No. 17 can be considered to have no genotoxic potential and additional tests are unnecessary.

Carcinogenicity

No study was reported.

Toxicokinetics

No data were reported.

Human data

No data were reported.

4. CONCLUSION

(1) In light of the data provided, does the SCCS consider the new hair dye HC Red No. 17 (B120) safe when used in non-oxidative hair colouring products at concentrations of up to 0.5%?

The SCCS considers the hair dye HC Red No. 17 safe when used in non-oxidative hair colouring products at concentrations up to 0.5%.

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Red No. 17 (B120) in cosmetic products?

HC Red 17 is a tertiary amine. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb.

5. MINORITY OPINION

/

6. REFERENCES

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