Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Red n° 16

COLIPA n° B114

The SCCS adopted this opinion at its 9th plenary meeting on 14 December 2010

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.

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).

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.).

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This opinion has been subject to a commenting period of four 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.

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1. BACKGROUND

Submission I of HC Red n° 16 with the chemical name: N-(2-nitro-4-aminophenyl)-allylamine (B114) was submitted by COLIPA [1] in July 1998 [2].

Submission II was submitted by COLIPA in July 2005. According to this submission HC Red n° 16 is used as a direct hair dye for hair colouring products (final concentration on head 1.5%). It can also be used in oxidative hair dye formulations with and without mixing with an oxidising agent (e.g. hydrogen peroxide) with a final concentration of 0.75% on the head.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Safety (SCCS) consider HC Red n° 16 safe for use as non-oxidative hair dye formulations with a concentration of maximum 1.5% on the head taken into account the scientific data provided?*

2. *Does the SCCS consider HC Red n° 16 safe for use in oxidative hair dye formulations with a concentration of 1.5% in the finished cosmetic products resulting in a concentration of 0.75% on the head after mixing with an oxidising agent taken into account the scientific data provided?*

3. *Does the SCCS recommend any restrictions with regard to the use of HC Red n° 16 in oxidative or non-oxidative hair dye formulations (e.g. max conc. in the finish cosmetic product, dilution ratio with hydrogen peroxide, warning etc?)*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

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 n° 16 (INCI name)

3.1.1.2.	Chemical names

1,4-Benzenediamine, 2-nitro-N1-2-propenyl- (9CI)
N-(2-Nitro-4-aminophenyl)-allylamine

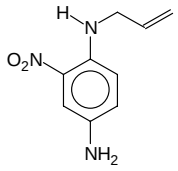
3.1.1.3	Trade names and abbreviations

Ro 1318
COLIPA B 114

3.1.1.4	CAS /EC number

CAS: 160219-76-1
EC: / (substance not in use in EU)

3.1.1.5	Structural formula



3.1.1.6	Empirical formula

Formula: $C_9H_{11}N_3O_2$

3.1.2	Physical form

Dark brown-black crystals

3.1.3	Molecular weight

Molecular weight: 193.2 g/mol

3.1.4	Purity, composition and substance codes

Batch Ro-EI 5040/24

Identity: verified by NMR-spectroscopy, elemental analysis
Purity by NMR assay: 94% (w/w)
Purity by HPLC assay: > 98% (peak area)

Impurities: 1.2% (w/w) 4-Fluoro-3-nitroaniline
Total fluorine content: 0.2% (w/w)

Ref.: 2

Batch Ro-El 5622/134 = SAT 950726

Identity: verified by elemental analysis and the retention time in TLC - and HPLC chromatograms
Purity by HPLC assay: 99.2% (peak area)
Impurities: 0.7% (w/w) 4-Fluoro-3-nitroaniline
Total fluorine content: 0.1% (w/w)

Ref.: 3

Batch Ro-RN6794-107 = SAT 040282

Identity: verified by NMR-spectroscopy, IR-spectrometry and UV-spectrometry
Purity by NMR assay: 98.6% (w/w)
Purity by HPLC assay: 98.7% (peak area)
Sulfated ash content: <0.1% (w/w)
Impurities:
Solvent content (water): < 0.1% (w/w)
0.19% (w/w) 4-Fluoro-3-nitroaniline
4-hydroxy-3-nitroaniline, not detected

Ref.: 4

Other impurities: Pb < 20 ppm, Sb and Ni < 10 ppm, As and Cd < 5 ppm, Hg < 1 ppm

Ref.: 1

Comment
HPLC identification and purity measurement were performed at 246 nm, but not at the specific wavelength (505 nm)

Declaration concerning HC Red n° 16 used in the marketed products

Purity by NMR assay: > 98.0% (w/w)
Purity by HPLC assay: > 98.0% (area)
Solvent content (water): < 0.5% (w/w)
Impurities: 4-Fluoro-3-nitroaniline < 0.5% (w/w)

3.1.5 Impurities / accompanying contaminants

See 3.1.4.

3.1.6 Solubility

Water:	< 1 g/l	room temperature
Ethanol:	5 – 50 g/l	room temperature
DMSO:	> 100 g/l	room temperature

Comment
Method for water solubility determination is not reported.

3.1.7	Partition coefficient (Log P_{ow})

Log $P_{o/w}$: 1.4 (calculated)

Comment
Log $P_{o/w}$ has not been determined by EEC method A.8.

3.1.8	Additional physicochemical specifications

Melting point:	77 – 84 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV/Visible spectrum:	λmax at 247nm and 505 nm

3.1.9.	Stability

Stability of HC Red n° 16 in various test formulations is not reported. The stability under oxidative conditions was not reported.

General Comments on Physico-chemical characterisation

- HC Red n° 16 is not registered under the chemical legislation in the EU.
- HPLC identification and purity measurement was performed at 246 nm, but not at the specific wavelength (505 nm)
- The Log P_{ow} strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log P_{ow}, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
- The stability of HC Red n° 16 in various test formulations and in typical hair dye formulations is not reported. The stability under oxidative conditions was not demonstrated.
- HC Red n° 16 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

3.2. Function and uses

HC Red n° 16 is used as a direct hair dye in hair colouring products. It can also be used in oxidative hair dye formulations with and without mixing with an oxidising agent (e.g. hydrogen peroxide).

The final concentration on head of HC Red n° 16 can be up to 1.5% when it is used without an oxidising agent and up to 0.75% after mixing with an oxidising agent.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Rat, Sprague-Dawley
Group size:	40 (20 males and 20 females)
Test substance:	HC Red n°16 (Sat 950726)
Batch:	Ro-EL 5622/134
Purity:	99.2%
Vehicle:	1% aqueous dispersion of carboxymethylcellulose in deionised water
Dose:	200, 316 and 501 mg/kg bw (5 males and 5 females per group)
Dosage volumes:	20 ml/kg bw
Route:	oral gavage
Observation period:	2 weeks
GLP:	in compliance
Study date:	1995

Results
One female was found dead 15 minutes after treatment at the dose of 316 mg/kg bw. At the dose level of 501 mg/kg bw, 3 males and one female were found dead on day 2, one additional male and four females were found dead on day 3.
Signs of reactions to treatment were observed shortly after dosing in all surviving animals, including lethargy, red discolorations of urine and red teguments.
No body weight changes comparing to control rats were noted in the low dose group and in the females from the medium dose group. Males treated at 316 mg/kg bw showed a statistically significant delay of body weight gain from day 8 onwards.
Autopsy of the rats found dead during the study revealed discoloration of the stomach with black areas, marked dilatation of the bladder by a black liquid, pale white to dark red areas in the liver.
Recovery of survivors, as judged by external appearance and behaviour, was apparently complete within 4 days of treatment. No macroscopic findings that could be associated with treatment were observed on rats sacrificed on study termination.

Conclusion
The acute median lethal oral dose (LD_{50}) to rats of HC Red n°16 was calculated to be around 392 mg/kg. HC Red n°16 should then be considered as harmful when administered by the oral route.

Ref.: 5

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species/strain:	Male New Zealand White rabbit
Group size:	3 animals
Test substance:	HC Red n°16
Batch:	Ro-El 5040/24
Purity:	>98%
Dose:	0.5 g moistened test substance
Observation period:	4 days
GLP:	yes
Study date:	1993

The acute dermal irritation properties of HC Red n°16 were investigated in healthy adult male albino rabbits of the New Zealand White strain. Approximately 24 hours prior to the treatment, the dorsal fur was shaved to expose an area of about 150 cm². An aliquot of 0.5 g of the moistened test substance was exposed to the intact shaved back skin of each animal. The patch was removed four hours after semi-occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approx. 1 hour, 24, 48 and 72 hours after termination of the exposure.

Results
Under the conditions of the study, the undiluted test substance was neither irritating nor corrosive when applied to the intact rabbit skin under semi-occlusive patch conditions.
The animals did not show any symptoms of systemic intoxication.

Conclusion
HC Red n°16 is non irritating when applied to the intact rabbit skin.

Ref.: 6

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species/strain:	Male New Zealand White rabbit
Group size:	3
Test substance:	HC Red n°16
Batch:	Ro-El 5040/24
Purity:	>98%
Dose:	71 ± 1 mg/animal
Observation period:	8 days
GLP:	yes
Study date:	1993

The acute eye irritation properties of undiluted HC Red n°16 were investigated.
The equivalent of 1 ml of HC Red n°16 (71 ± 1 mg) was instilled into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls.
The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours and 7 days after instillation of the test solution.

Results

The instillation of undiluted HC Red n°16 into the eyes resulted in slight redness of the conjunctivae. The irritation of the conjunctivae had resolved within 48 hours in two animals and within 7 days after instillation in the third animal.
Treatment of the eyes with 2% fluorescein, 24 hours after test substance instillation revealed no corneal epithelial damage in any of the animals.
There was no evidence of ocular corrosion and the animals did not show any symptoms of systemic intoxication.

Conclusion
Undiluted HC Red n°16 was slightly irritating to the rabbit eye.

Ref.: 7

3.3.3. Skin sensitisation

LLNA

Guideline:	OECD 429
Species/strain:	female CBA/CaOlaHsd mice
Group size:	4 /group
Test substance:	HC Red n°16
Batch:	Ro-RN6794-107
Purity:	99.4%
Dose levels:	5, 10 and 25%
Vehicle:	acetone:olive oil (4:1 v/v)
Route:	topically
Radiochemical:	thymidine (^{3}HTdR)
Positive control:	α-hexylcinnamaldehyde
GLP:	yes
Study date:	July 2004

The dermal sensitization properties of HC Red n°16 were investigated in healthy mice of the CBA/CaOlaHsd strain. Three dose groups and a control group (receiving the vehicle only) of 4 female mice each were chosen. The test item was topically applied to the dorsal surface of the ears to analyse the sensitisation activity by measuring the proliferative response of lymph node cells.
A homogenous dilution of the test item in a mixture of acetone:olive oil (4:1 v/v) was made shortly before each dosing. The highest technically applicable test item concentration of 25% was found to be non-irritating in a pretest with two female mice. Based on these test results 5%, 10% and 25% solutions were chosen for the main study.
Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}HTdR) by intravenous injection via the tail vein.
Approximately five hours after ^{3}HTdR application all mice were euthanized. The draining lymph nodes were excised and pooled. The level of ^{3}HTdR incorporation was then measured by liquid scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of ^{3}HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).
The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methyl thymidine. The stimulation index (S.I.) was calculated.

Results
All treated animals survived the scheduled study period.

No clinical signs were observed in any animals of the control group and the low and mid dose group. On the second application day, all mice of the high dose group excreted deep yellow urine, persisting for a total of three days.
The Stimulation Index (S.I.) was above 3 in all dose groups. No dose response relation was noted.

Test Item Concentration	S.I.
5% (w/v)	3.0
10% (w/v)	3.9
25% (w/v)	3.8
Positive control	**S.I.**
5% (w/v)	1.5
10% (w/v)	2.3
25% (w/v)	8.4

The EC3-value can be considered to be about 5%.
The positive control showed an EC3 value of 11.7%.

Conclusion
HC Red n°16 was found to be a moderate sensitizer when tested in acetone:olive oil (4:1) in mice.

Ref.: 8

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428
Tissue:	pig skin (2 animals)
Group size:	two experiments with four replicates (eight chambers in total)
Skin integrity:	TER
Diffusion cell:	static Franz diffusion cells
Test substance:	HC Red n°16
Batch:	Ro-RN6794-107
Purity:	99.4%
Test item:	A: HC Red n°16 at 1.5% in a direct dye cream B: HC Red n°16 at 0.75% final concentration in an oxidative dye cream mixed with hydrogen peroxide C: HC Red n°16 at 1.5% final concentration in 50% aqueous ethanol solution
Dose:	20 mg formulation per cm²
Dose of test substance:	0.3 mg/cm² skin in experiment A and C and 0.15 mg/cm² in experiment B.
Receptor fluid:	Dulbecco's phosphate buffered saline (pH 7.35)
Solubility receptor fluid:	in water < 1g/L
Stability receptor fluid:	
Method of Analysis:	HPLC
GLP:	yes
Study date:	2005

The dermal absorption/percutaneous penetration of HC Red n°16 from two standard hair dyeing formulations and an aqueous ethanol solution was studied on the clipped excised skin of two young pigs. The pig skin, dermatomed to a mean thickness of 0.75 mm, was used.
The skin integrity of frozen (at -20 °C) skin discs was checked by measuring the trans-dermal electrical resistance. The intact, clipped excised pig skin of the flanks area was exposed for 30 minutes to the test substance in the basic hair dyeing formulation without occlusion.

The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 20 mg formulation per cm² pig skin, resulting in a dose of the test substance of approx. 0.3 mg/cm² skin in experiment A and C and 0.15 mg/cm² in experiment B. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 80 solution and water.
Each of the two formulations and the solution were analysed in two experiments with four replicates per experiment for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was Dulbecco's phosphate buffered saline (pH 7.35). In the static system, samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 1, 2, 4, 6, 24, 29 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Results
The quantities that had penetrated during the 30 minute exposure to COLIPA B114 containing formulations and within the 48 hours after application are shown in the following table. Both the amounts absorbed and penetrated were taken as systemically available.

ANALYSED SAMPLE	Formulation A direct dye cream without H_2O_2		Formulation B oxidative dye cream with H_2O_2		Solution C aqueous ethanol solution	
	[% of dose]	[µg/cm²]	[% of dose]	[µg/cm²]	[% of dose]	[µg/cm²]
Skin rinsings	84.6	-	96.0	-	71.7	-
Adsorption (stratum corneum)	0.21	0.63	0.14	0.21	4.59	14.54
Not Bioavailable	84.8	-	96.1	-	76.3	-
Absorption (epidermis/dermis)	0.38	1.17	0.41	0.64	1.61	5.08
Penetration (receptor fluid)	2.81	8.43	1.27	1.84	3.65	11.55
Bioavailable	3.19	9.60	1.67	2.48	5.26	16.63
Total recovery / mass balance	88.1	-	97.8	-	82.5	-

In conclusion, in this *in vitro* dermal penetration study the amount of HC Red n°16 systemically available from a standard direct dye cream formulation was found to be 9.60 ± 4.96 µg/cm² (3.19 ± 1.40%) and from a oxidative dye cream mixed with a hydrogen peroxide containing developer 2.48 ± 1.02 µg/cm² (1.67 ± 0.55%), respectively.

Ref.: 16

Comment
The skin of only two pigs was used. 4.52 µg/cm² (the mean + 2SD) will be used to calculate the MOS under oxidative conditions and 19.52 µg/cm² (the mean + 2SD) under non-oxidative conditions

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline: OECD 408
Species/strain: Rat, Sprague-Dawley [Ico:OFA.SD. (IOPS Caw)]

Group size:	100 (10 males and 10 females per doses) + 40 (5 males and 5 females per dose except for the 40 mg/kg bw dose group) for the recovery study
Test substance:	HC Red n°16 (Sat 950726)
Batch:	Ro-EL 5622/134
Purity:	99.3%
Vehicle:	1% aqueous dispersion of carboxymethylcellulose in deionised water
Dose:	0, 10, 30, 40, 60 mg/kg bw
Dosage volumes:	5 ml/kg bw
Route:	oral gavage
Study period:	13 weeks + 4 weeks for the recovery group
GLP:	in compliance
Study date:	November 1995 - June 1997

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 10, 30, 40 and 60 mg/kg bw/day, once daily for 13 weeks. During the study, the animals were observed twice daily for clinical signs and mortality, and weekly for full clinical examination, body weight and food and water consumption. Clinical pathology investigations were performed after 4 weeks of treatment and at the end of the treatment and treatment-free periods. Ophthalmological examination was conducted before the start of the study and at the end of the treatment period on control and high dose animals. At the end of the treatment period, a full autopsy was conducted with recording of weights of the adrenals, liver, kidney, brain and testes and macroscopic and microscopic examination of major organs. In addition a detailed examination of the stomach (including duodenum) was performed and photographs of the stomach of each animal were taken. A microscopic examination of the brain (cerebrum and cerebellum) and sciatic nerve was performed in the animals from the control and high dose groups killed at the end of the treatment period.

Results
One male and one female from the high dose group were found dead before the end of the study. The authors considered that the cause of death was probably a gavage error.
From day 1 or 2 of the study, rats from all treated groups passed coloured urine.
For most of the treated animals, the administration of the test substance was associated with a characteristic pattern of clinical signs: erect tails, hypertonus and pedalling behaviour, soon after dosing, suggesting a neurological effect of the treatment. These changes were more marked in females and reversible during the treatment-free period. The incidence and time of onset from the start of treatment were dose-related.
Microscopic examination of the brain and sciatic nerves revealed no corresponding lesion although the serum cholinesterase activities of the high dose group females were decreased in week 13.
The body weight gain and food consumption were comparable for all dose groups.
Only minor differences in haematological parameters and in biochemical parameters were observed. They were not dose-related and reversible and, therefore, not considered to be of toxicological significance.
No abnormal findings were reported in the ophthalmological examinations.
The liver weight of rats was increased in all treated females in a dose dependent way. The kidney weights of females of high dose group were higher than in controls. The organ weight changes were not observed in rats killed at the end of the recovery period. The increase in liver weight may suggest increased metabolism due to test article elimination.
Macroscopic changes were noted in the stomachs of both treated and control animals from both sexes at termination and at the end of the treatment-free period and included: depressed areas, raised areas, pale or dark areas, thickened wall, streaks and adhesions to liver.
Microscopic examination of the tissues (excluding sciatic nerve and brain) from the controls and high dose group animals revealed no treatment-related lesions. Histological examination of brain and sciatic nerves showed no abnormality.

Conclusion
Oral gavage of HC Red n°16 to the rat at dose levels between 10 and 60 mg/kg bw/d for 13 weeks was associated with a range of clinical signs suggestive of a neurological effect. These signs included erect tails, hypertonus and pedalling behaviour.

Based on these neurological effects, a clear NOEL was not determined in the study.

Ref.: 13

In order to obtain more information for the interpretation of behavioural changes of the rats, a further group of 5 female Sprague Dawley rats (the more sensitive sex for neurological effects based on the previous experiment), was administered orally by stomach intubation with 50 mg/kg bw/d HC Red n° 16 for 90 days. The concentrations of HC Red n°16 in blood and brain were determined 24h after the last administration. Special attention was given to the occurrence of the following signs: hypersalivation, pedalling behaviour, rigid posture and erected tail.
All treated rats survived during the study. Discoloured urine was noted almost every day in all animals. This finding is not considered as an adverse effect. Reduced motor activity was also noted frequently after oral administration but was considered as a consequence of stress induced by the gavage. Hypersalivation, pedalling behaviour, rigid posture and erected tail were not observed at all or to an extent that would not justify a relation to the test substance.
In blood, a mean concentration of 26.8 µg equivalents of the test substance per gram was detected. In the different brain section, the means ranged from 2.39 to 3.66 µg equivalents of the test substance per gram. The highest concentrations were found in the brain stem.

Based on the results of this study and the previous one, the applicant concluded that HC Red n° 16 induced no systemic cumulative toxic effect when administered in a daily dose of up to 60 mg/kg bw/d in rats. A NOAEL of 50 mg/kg bw/ d is proposed.

Ref.: 14

Comment
The results of the forelimb grip strength performed in this study are not interpreted; there is no control group and only one dose tested. This study is also performed in another facility than the previous one. The results of this study cannot overrule those observed in the reference 13.
Based on the neurological effect observed in the 13 weeks toxicity study, 10 mg/kg bw/d should be considered as a LOAEL and used for the calculation of the margin of safety.

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 Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537.
Replicates:	triplicate in a single experiment
Test substance:	B114
Batch:	RO-RN6794-107
Purity:	99.4% (area %, HPLC)
Solvent:	DMSO

Concentrations: 33, 100, 333, 1000, 2500 and 5000 µg/plate, with and without S9-mix
Treatment: plate incorporation test, with and without S9-mix
GLP: in compliance
Study period: June 2004 – September 2004

B 114 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity and mutation in a pre-experiment with strains TA98 and TA100 both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or thinning of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in both strains used the pre-experiment is reported for these 2 strains as main experiment. The main experiment was performed with the plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
The plates incubated with B 114 showed normal background growth up to 5000 µg/plate without and with S9-mix in all strains used. Toxic effects evident as a reduction in the number of revertants were observed for TA1537 at 5000 µg/plate and for TA102 at 1000 - 5000 µg/plate without and with S9-mix.
In the presence of S9-mix treatment with B 114 resulted in a biologically relevant and more or less dose dependent increase in revertant colonies in TA102 and TA1535. At the higher concentrations the number of colonies was reduced due to overlapping toxic effects.

Conclusion
Under the experimental conditions used B 114 was genotoxic (mutagenic) in this gene mutation tests in bacteria.

Ref.: 9

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y $tk^{+/-}$ mouse lymphoma cells	
Replicates:	duplicate cultures in two independent experiments	
Test substance:	B 114	
Batch:	Ro-RN6794-107	
Purity:	99.4% (area %)	
Solvent:	DMSO	
Concentrations:	Experiment I:	31.3, 62.5, 125, 250, 375 and 500 µg/ml without S9-mix 3.8, 7.5, 15, 30, 60 and 90 µg/ml with S9-mix
	Experiment II:	7.5, 15, 30, 60, 90 and 120 µg/ml without S9-mix 1.9, 3.8, 7.5, 15, 22.5 and 30 µg/ml with S9-mix
Treatment	Experiment I:	4h with and without S9-mix; 72h expression period and a selection period of 10-15 days.
	Experiment II:	24h without S9-mix; 48h expression period and a selection period of 10-15 days. 4h with S9-mix; 72h expression period and a selection period of 10-15 days.
GLP:	in compliance	
Study period:	9 November 2004 – 6 April 2005	

B 114 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. The highest

concentration used in this pre-test was 2000 µg/ml (≈ 10mM, the prescribed recommended dose). In the main test, cells were treated for 4 h or 24 h (without S9-mix experiment II) followed by an expression period of 72 or 48 h (without S9-mix experiment II) to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as relative total growth compared to the relative total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
No precipitation of B 114 was observed with the unaided eye in the main experiments up to the maximum concentrations. The recommended toxic range of approximately 10-20 % survival compared to the concurrent negative controls was covered in all experiments.
When tested up to toxic concentrations in both experiments a biologically relevant increase in mutant frequency compared to concurrent controls was not observed neither in the absence nor in the presence of S9-mix.
The threshold of twice the mutant frequency of the corresponding solvent control was exceeded at 15 and 30 µg/ml in the second culture of experiment I with S9-mix and at 120 µg/ml in the second culture of experiment II without S9-mix. However, the absolute values remained well within the historical range of negative and solvent controls. An increase of the mutant frequency exceeding the historical negative and solvent controls range occurred at 30 µg/ml in the first culture of experiment I with S9-mix. However, this increase was not reproducible in the other culture and the other experiment. These occasional findings were judged to be caused by secondary (toxic) effects and considered without biological relevance.

Conclusion
Under the experimental conditions used, B 114 did not induce mutations at the *tk* locus of L5178Y mouse lymphoma cells and, consequently, is not genotoxic (mutagenic) in the gene mutation test.

Ref.: 10

In vitro micronucleus test in V79 cells

Guideline:	OECD 473 (1997; *In vitro* mammalian chromosome aberration test)
Cells:	Chinese hamster V79 cells
Replicates:	duplicate cultures in a single experiment
Test substance:	B 114
Solvent:	DMSO
Batch:	Ro-RN6794-107
Purity:	99.4 % (area %, HPLC)
Concentrations:	93.8, 187.5 and 375 µg/ml without S9-mix 46.9, 93.8 and 187.5 µg/ml with S9-mix
Treatment	4 h treatment with and without S9-mix; harvest time 24 hours after the beginning of treatment
GLP:	In compliance
Date:	21 September 2004 – 3 February 2005

B 114 was investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. A pre-test on cell growth inhibition (XTT assay) with 4 h treatment was performed in order to determine the toxicity of B 114, the solubility during exposure and changes in osmolarity and pH value at experimental conditions. Dose selection was performed following the current OECD Guideline 473 for chromosomal aberration studies. With respect to the molecular weight of B 114, 2000 µg/ml (≈ 10mM, the prescribed recommended dose) was

applied as top concentration for treatment of cultures in this pre-test. Using the XTT activity of about 40% of control or below as an indicator for toxicity in the pre-test and considering the data from the pre-test, the formulability of B 114 and occurrence of precipitates in the culture medium a top concentration in the main test of 1500 µg/ml without and with S9-mix was chosen.
The treatment period in the main test was 4 h without and with S9-mix. Harvest time was 24 hours after the beginning of culture. To describe a toxic effect and thus exposure the relative cell count and the XTT activity of the test groups were determined as reduction of cells (in %) as compared to the respective solvent control. Concurrent negative and solvent controls were employed.

Results
Due to strong precipitation and toxicity, the top concentrations evaluated in the main tests were 375 µg/ml and 187.5 µg/ml without and with S9-mix, respectively. Precipitation of B 114 in culture medium was observed at 178.5 and 375 µg/ml without S9-mix and at 178.5 µg/ml with S9-mix. No relevant increase in the osmolarity or change in pH value was observed.
Both in the absence and in the presence of S9-mix, biologically relevant, dose dependent and statistically significant increases in the percentage of V79 cells with micronuclei were observed.

Conclusion
Under the experimental conditions used, B 114 did induce an increase in V79 cells with micronuclei in vitro both in the presence and absence of metabolic activation and, consequently is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.: 11

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Bone marrow micronucleus test in mouse

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mouse
Group size:	5 mice/sex/group
Test substance:	B 114
Batch no:	Ro-RN6794-107
Purity:	99.4 %
Dose level:	0, 62.5, 125 and 250 mg/kg bw
Route:	intraperitoneal injection
Vehicle:	Corn oil
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	in compliance
Date:	21 December 2004 – 4 March 2005

B 114 was investigated for the induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a dose range finding study on acute toxicity, mortality and physical condition. Two male and 2 female mice were treated with 250 and 500 mg/kg bw and examined at 30 and 90 min as well at day 2 and 3 after treatment. In the main experiment mice were exposed orally to 0, 62.5, 125 and 250 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Additional animals were dosed with the highest dose and the vehicle for blood sampling. Blood was sampled 1 and 4 h after treatment to analytically demonstrate the bioavailability of B 114 in case that the exposure could not be demonstrated by severe toxic effects seen during the study. Negative and positive controls were in accordance with the OECD guideline.

Results
During the first hour after dosing all mice were lethargic. The mice treated with 125 and 250 mg/kg bw had their hairless body parts coloured red and several mice demonstrated ataxia and ventral recumbency as well. Within 21 h after dosing the mice of the 250 mg/kg bw group showed a rough coat and several demonstrated a hunched posture. At 21 h after treatment the mice treated with 62.5 and 125 mg/kg bw and at 45 h after dosing the mice treated with 250 mg/kg bw had recovered from treatment.
After treatment with B 114 the ratio PCE/NCE was not substantially decreased as compared to the mean ratio of the vehicle control, thus indicating that B 114 did not exert any cytotoxic effects in the bone marrow. However, the systemic toxic signs of the treated animals observed after treatment indicated the systemic distribution of B 114 and thus its bioavailability. The bioavailability of B 114 was also demonstrated by the determination of B 114 in blood samples.
Biologically relevant or statistically significant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment and neither for males nor for females.

Conclusion
Under the experimental conditions used B 114 did not induce a biologically relevant increase in the number of PCEs with micronuclei of treated mice and, consequently, B 114 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 12

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

Prenatal developmental study

Guideline:	OECD 414
Species/strain:	rat, Sprague-Dawley [Ico:OFA.SD. (IOPS Caw)]
Group size:	100 (25 females per group)
Test substance:	HC Red n°16 (SAT 950726)
Batch:	Ro-EL 5622/134
Purity:	99.3%
Vehicle:	1% aqueous dispersion of carboxymethylcellulose in deionised water
Dose levels:	0, 30, 40 and 60 mg/kg bw/d
Dose volume:	5ml/kg bw
Route:	gavage
Administration:	once daily during day 6 to day 15 of gestation
GLP statement:	in compliance
Study date:	January 1996

100 pregnant rats (25 per group) of the Sprague-Dawley strain were treated once daily by oral gavage of HC Red n° 16 in 1% aqueous dispersion of carboxymethylcellulose in deionised water day 6 to day 15 of gestation at the doses of 0, 30, 40 and 60 mg/kg bw/d. The animals received a constant volume of 5 ml/kg bw/d. The test procedure followed the OECD guideline and was conducted in compliance with the principles of GLP.

During the study the mortality, signs of intoxication, body weight and food consumption were recorded. All mated females were sacrificed on day 20 of gestation. In the pregnant females, a macroscopic examination of the organs was carried out. The number of alive and dead foetuses, their distribution and site in uterus, early and late resorptions, implantations and number of corpora lutea were determined. The weight of the foetuses, gravid uteri, uteri without foetuses, placentae and the sex of foetuses were recorded. All foetuses were weighed, sexed and examined for external abnormalities. Half of the foetuses were then examined viscerally prior to evisceration and the remaining foetuses were examined for skeletal malformations, variations and retardations of the normal organogenesis after appropriate staining.

Results
No rats died during the treatment period. No toxic effects were reported during the study. Female of all dose groups had red discoloured urines during the last 4 days of treatment period at dose related intensity.
There was a slight but statistically significant dosage-related reduction in mean body weight gain in all treated groups compared with the control from G6 (first day of treatment) until G11 of pregnancy.
There was a slight but statistically significant reduction in food consumption gain in all treated groups compared with the control from G6 (first day of treatment) until G11 of pregnancy, although there was no obvious dosage relationship.
Gross necropsy did not reveal any organ alterations related to treatment.
No significant differences in the number of viable foetuses, the male to female sex ratio, birth- position, number of runts, post-implantation losses, implantations, resorptions and corpora lutea between dosage groups and the control group were observed.
There were no foetuses classified as malformed in any of the groups and no visceral anomalies considered to be related to treatment. There were no treatment-related visceral or skeletal findings noted at the fixed foetal examinations.

Conclusion
There was evidence of maternal toxicity in all the treated groups which included reduced body weight gain and food consumption. However, there was no obvious evidence of an adverse effect of treatment on the developing foetuses at dosages up to 60 mg/kg bw/day.
Ref.: 15

Comment
The Lowest Observed Effect Level (LOEL) of HC Red n° 16 in female rats after daily oral treatment is 30 mg/kg bw/day. The NOAEL of embryo/foetal toxicity is 60 mg/kg bw/day, the highest dose tested.

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

HC Red n° 16

Non oxidative conditions

Absorption through the skin	**A (mean + 2SD)**	**=**	**19.52 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**11.32 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.19 mg/kg bw/d**
LOAEL (90-day, rat, oral)	**LOAEL**	**=**	**10 mg/kg bw/d**
Adjusted by factor 3		**=**	**3.33**
MOS		**=**	**18**

HC Red n° 16

Oxidative conditions

Absorption through the skin	**A (mean + 2SD)**	**=**	**4.52 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.62 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.04 mg/kg bw/d**
LOAEL (90-day, rat, oral)	**LOAEL**	**=**	**10 mg/kg bw/d**
Adjusted by factor 3		**=**	**3.33**
MOS		**=**	**83**

3.3.14. Discussion

Physico-chemical specification
HC Red n° 16 is used as a direct hair dye for hair colouring products. It can also be used in oxidative hair dye formulations with and without mixing with an oxidising agent (e.g. hydrogen peroxide). The final concentration on head of HC Red n° 16 can be up to 1.5%

when it is used without an oxidising agent and up to 0.75% after mixing with an oxidising agent.
HPLC identification and purity measurement was performed at 246 nm, but not at the specific wavelength (505 nm). The Log P_{ow} strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log P_{ow}, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
Stability of HC Red n° 16 in various test formulations and in typical hair dye formulations is not reported. The stability under oxidative conditions was not demonstrated.
HC Red n° 16 is not yet registered in EU.
HC Red n° 16 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

General toxicity
The acute median lethal oral dose (LD_{50}) to rats of HC Red n° 16 was calculated to be around 392 mg/kg.

Oral gavage of HC Red n°16 to the rat at dose levels between 10 and 60 mg/kg bw/d for 13 weeks was associated with a range of clinical signs suggestive of a neurological effect. These signs included erect tails, hypertonus and pedalling behaviour.

Based on the neurological effects observed in the 13 weeks toxicity study, a clear NOAEL can not be determined and 10 mg/kg bw/d was considered as a LOAEL and used for the calculation of the margin of safety.

Based on a oral prenatal developmental study on rat showing maternal toxicity including reduced body weight gain and food consumption, a maternal LOAEL of 30 mg/kg bw/day and a NOAEL of embryo/foetal toxicity of 60 mg/kg bw/day (highest dose tested), could be determined.

Irritation, sensitisation
The neat substance was not irritating to rabbit skin and slightly irritating to the rabbit eye. HC Red n° 16 is a moderate sensitizer when tested in acetone:olive oil.

Dermal absorption
In an *in vitro* dermal absorption assay using pig skin, the amount of HC Red n° 16 systemically available from a standard direct dye cream formulation was 9.60 ± 4.96 µg/cm² (3.19 ± 1.40%) and 2.48 ± 1.02 µg/cm² (1.67 ± 0.55%) from a oxidative dye cream mixed with a hydrogen peroxide containing developer. As skin of only two pigs was used in the experiment, the mean value + 2SD was used to calculate the MOS. The large difference in dermal absorption between oxidative and non-oxidative conditions may indicate that HC Red n° 16 is not stable under oxidative conditions.

Mutagenicity
Overall, the genotoxicity of HC Red n° 16 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Red n° 16 was mutagenic in bacteria but not in an *in vitro* gene mutation test in mammalian cells. HC Red No. 16 induced an increase in cells with micronuclei in an *in vitro* micronucleus test.

The putative clastogenic potency of HC Red n° 16 could not be confirmed in a mouse bone marrow micronucleus tests. However, an *in vivo* gene mutation assay to investigate whether the mutagenic potency of HC Red n° 16 found in the gene mutation test in bacteria

also appears in experimental animals was not conducted. Consequently, a definite conclusion on the mutagenicity of HC Red n° 16 can not be given.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the low margin of safety for the use in both oxidative and non-oxidative hair dye formulations, the SCCS is of the opinion that the use of HC Red n° 16 as a hair dye ingredient up to a final on-head concentration of 0.75% under oxidative and 1.5% under non-oxidative conditions poses a risk to the health of the consumer.

A definite conclusion on the mutagenicity of HC Red n° 16 cannot be drawn.

Data on the stability in an oxidative environment should be provided.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. Meinigke, B. (2005). Raw material description – B 114. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500192
2. Meinigke, B. (2005). Dossier of hair dye B114 – analysis of batch Ro-El 5040/24 used in toxicological tests. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500238
3. Meinigke, B. (2005). Dossier of hair dye B114 – analysis of batch Ro-El 5622/134 used in toxicological tests. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500221
4. Meinigke, B. (2005). Dossier of hair dye B114 – analysis of batch RO-RN 6794-107 used in toxicological tests. Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500193
5. Mercier, O. (1996). SAT 950726 – single dose toxicity study by the oral route in the rat (LD_{50}). Pharmakon Europe, L'Arbresle Cedex, France, internal study code: 42995. Archive code at Henkel KGaA, Düsseldorf, Report No. R 9600188
6. Rijcken, P. (1993) Primary skin irritation / corrosion study with Ro 1318 in the rabbit (4-Hour Semi-Occlusive Application) NOTOX B.V., s'-Hertogenbosch, The Netherlands, internal study code: 099877 Archive code at Henkel KGaA, Düsseldorf, Report No. R 9300188
7. Rijcken, P. (1993) Acute eye irritation / corrosion study with Ro 1318 in the rabbit NOTOX B.V., s'-Hertogenbosch, The Netherlands, internal study code: 099888 Archive code at Henkel KGaA, Düsseldorf, Report No. R 9300179
8. Wang-Fan, W. (2004) B 114: Local Lymph Node Assay (LLNA) in mice (identification of contact allergens) RCC Ltd, Itingen, Switzerland, internal study code: 854142 Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400553
9. Sokolowski, A. (2004) *Salmonella typhimurium* reverse mutation assay with B 114 RCC Cytotest Cell Research GmbH, Rossdorf, Germany, internal study code 838720 Archive code at Henkel KGaA, Düsseldorf, Report No. R 0400963
10. Wollny, H.-E. (2005) Cell mutation assay at the thymidine kinase locus (TK+/-) in mouse lymphoma L5178Y cells with B 114, CCC Cytotest Cell Research GmbH,

Rossdorf, Germany, internal study code 861004, Archive code at Henkel KGaA, Report No. R 0500139

11. Schulz, M. (2005) *In vitro* micronucleus assay in Chinese hamster V79 cells with B 114, RCC Cytotest Cell Research GmbH, Rossdorf, Germany, internal study code 852102, Archive code at Henkel KGaA, Report No. R 0500072
12. Buskens, C.A.F. (2005) Micronucleus assay in bone marrow cells of the mouse with B 114 NOTOX B.V., s'Hertogenbosch, The Netherlands, internal study code 427769 Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500237
13. Pickersgill, N. (1997) SAT 950726 – 13 week oral (gavage) toxicity study in the rat followed by a 4 week treatment-free period. Pharmakon Europe, L`Arbresle Cedex, France, internal study code: 328/514, Archive code at Henkel KGaA, Düsseldorf, Report No. R 9700587
14. Vincze, E. and Hofer, H. (1997) Organ levels of "^{14}C labelled Ro-1318" in Rats Forschungszentrum Seibersdorf, Seibersdorf, Austria, internal study code: HE 44, Report No OEFZ-A-4079 Archive code at Henkel KGaA, Düsseldorf, Report No. R 9700524
15. Marsden, E.K.S. (1998) SAT 950726 - teratology study by oral route (gavage) in the rat (Segment II) Pharmakon Europe, L`Arbresle Cedex, France, internal study code: 328/516, Archive code at Henkel KGaA, Düsseldorf, Report No. R 9800075
16. Bornatowicz, N. and Hofer, H. (2005) "B 114": Dermal absorption/percutaneous penetration *in vitro* ARC Seibersdorf research GmbH, Seibersdorf, Austria, internal study code: HE95 Archive code at Henkel KGaA, Düsseldorf, Report No. R 0500225