Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Brown 17

COLIPA n° B007

The SCCS adopted this opinion at its 5th plenary meeting

of 24 March 2014

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 six 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, Basic Brown 17 (B007), Regulation 1223/2009, CAS 68391-32-2, EC 269-944-0

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Basic Brown 17 (B007), 24 March 2014, SCCS/1531/14, revision of 18 June 2014.

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1. BACKGROUND

Submission I, II, III and IV for Basic Brown 17 (B007) with the chemical name 8-[(4-Amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethyl-2-naphthalenaminium chloride (EC 269-944-0 and CAS 68391-32-2) and with the CAS 3070871-30-0 for the free base, was submitted respectively in 1992, in 2002, in 2006 and in 2010 by Cosmetics Europe[1].

The SCCS has evaluated the safety of Basic Brown 17 (B007) several times.
The first scientific opinion (SCCP/0683/04) was adopted by the Scientific Committee on Consumer Products (SCCP) in December 2004.
The second scientific opinion (SCCP/1173/08) was adopted on 30 September 2008.
The last scientific opinion (SCCS/1448/11, revision of 26/27 June 2012) on the use of the hair dyeing ingredient Basic Brown 17 (B007) was adopted by the Scientific Committee on Consumer Safety (SCCS) with the following conclusion:

The SCCS is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out. Before any further consideration, the following information must be submitted:
- a proper chemical specification of Basic Brown 17 with respect to its purity and impurities;
- data on the stability of Basic Brown 17 in the test solutions and in typical hair dye formulations;
- additional studies to exclude a genotoxic potential of Basic Brown 17.

The current submission V on the substance Basic Brown 17 is aimed to provide the requested information including two conducted in vitro genotoxicity assays. Basic Brown 17 is used in non-oxidative hair dye formulations in a concentration up to 2.0%.

2. TERMS OF REFERENCE

1. *Does SCCS consider Basic Brown 17 safe for use in non-oxidative hair dyes with a concentration of maximum 2.0% taken into account the scientific data provided?*

2. *And/or does the SCCS recommend any further restrictions with regard to the use of Basic Brown 17 in non-oxidative hair dye formulations?*

[1] Cosmetics Europe - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Basic Brown 17 (INCI)

3.1.1.2 Chemical names

2-Naphthalenaminium, 8-[(4-amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethyl-, chloride
8-[(4-amino-3-nitrophenyl)diazenyl]-7-hydroxy-N,N,N-trimethylnaphthalen-2-aminium chloride
8-[(4-Amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethyl-2-naphthalenaminium chloride
[8-[(4-amino-3-nitrophenyl)azo]-7-hydroxy-2-naphthyl]trimethylammonium chloride.
1-[(3-Nitro-4-aminophenyl)azo]-2-hydroxy-7-trimethylammoniumchloride, naphthalene-
1-(3'-Nitro-4'-amino)-phenyl-azo-2-hydroxy-7-trimethylammonium chloride, naphthalene-

3.1.1.3 Trade names and abbreviations

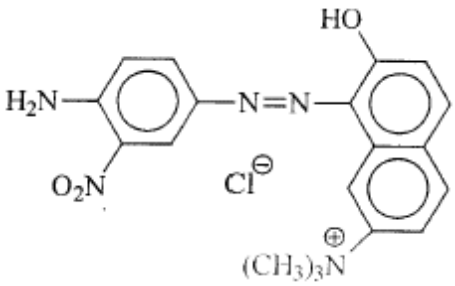
Synonym:	Arianor Sienna Brown, Jaracol Sienna, Sienna Brown
Sample codes of the applicant:	SAT 000918 SAT 040270 SAT 050019 Colour Index: CI 12251
COLIPA number:	B 007

3.1.1.4 CAS / EC number

CAS: 68391-32-2 (chloride)
EC: 269-944-0 (chloride)

Note
CAS n° 71134-97-9 and EC n° 275-216-3, which correspond to Basic Red 118 (8-[(4-Amino-2-nitrophenyl)azo]-7-hydroxy-2-naphthyl)trimethylammonium chloride, were used in the two previous COLIPA submissions for Basic Brown 17 (Submission I 1992 and Submission II 2001). This appeared to be due to an error in the EU cosmetic inventory, which has been corrected now.

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{19}H_{20}N_5O_3^+ Cl^-$ (Chloride content: 8.82%)

3.1.2 Physical form

Dark brown fine powder

3.1.3 Molecular weight

Molecular weight: 401.85 (as chloride)

3.1.4 Purity, composition and substance codes

Analytical description of Batches used in Toxicity studies

Batch	64960101 = SAT 050019 = SAT120004	NDKS 1944 = SAT 000918 = SAT 040270
Identity	verified by ^{1}H-, ^{13}C-NMR-spectroscopy, IR-spectrometry and UV-spectrometry	
Content by NMR	77.4% (w/w)	33% (w/w)
Purity by HPLC assay	94.2% (area) (230 nm) 98% (area) (460 nm)	96.3% (area)
		64.4% (w/w) *
Methylsulfate	11.6% (w/w)	8.2% (w/w)
Chloride	3.3% (w/w)	3.6% (w/w)
Sulfate	0.1% (w/w)	1.1% (w/w)
Sodium:	0.2% (w/w)	0.5% (w/w)
Saccharose **	--	15.1% (w/w)
Solvent content	7.0% (w/w)	6.5% (w/w)
water:	6.5% (w/w)	6.0% (w/w)
methanol:	0.5% (w/w)	0.5% (w/w)
Impurities:		
2-nitrobenzene-1,4-diamine	150 ppm	120 ppm
Basic Red 118 *** (2-nitro isomer of B17)	4.5% (w/w)	2.4% (w/w)
NBTRI	0.65% (w/w)	N.D.

* Batch 64960101 (NMR content 77.4%) was used as reference standard
** Note: batch NDKS 1944 represents an actual market material; it contains the extender saccharose which has been used to adjust the colour strength to a certain predefined value.
*** Basic Red 118 (CAS 71134-97-9) is banned as no safety file was submitted; however, an exception is included in entry 1291 of Annex II as a contaminant in Basic Brown 17 when used as a substance in hair dye products
NBTRI: 7-Hydroxy-N,N,N-trimethylnaphthalene-2-aminium chloride

Declaration of the applicant
"The batch of COLIPA B 007 used in the acute oral toxicity test is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From this information, it can be concluded that the former not fully described batch is representative and its specification is quite similar to the fully characterized batch 64960101."

SCCS comment
The dye content of batch SAT 050019 based on NMR, should be considered as semi-quantitative.
The dye content of batch NDKS 1944 based on HPLC analysis, should also be considered as semi-quantitative.

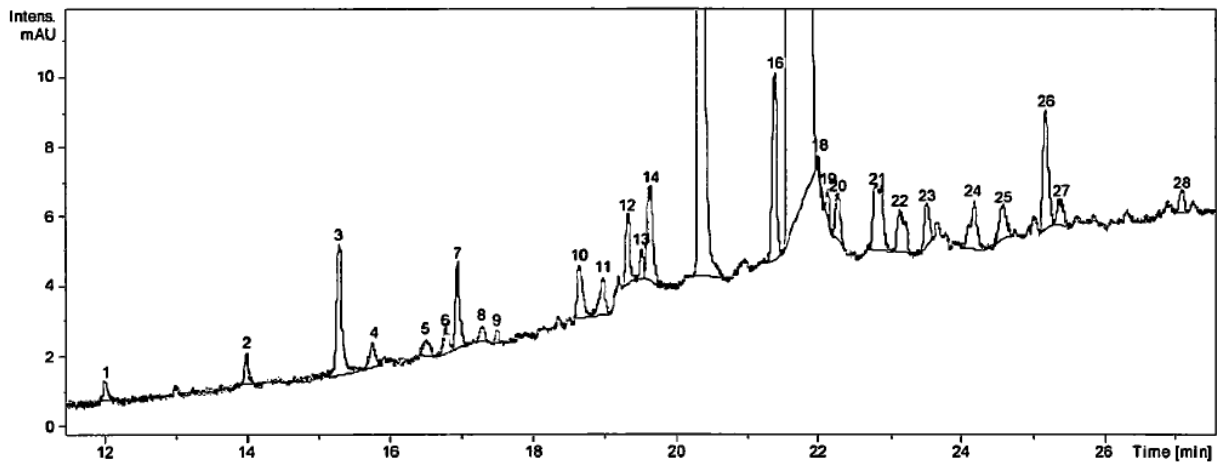
Declaration by the Applicant (Submission V)
Since Submission III (R 0500099), the quality of the product Basic Brown 17 has been improved in respect to a lower content of impurities, in particular to a lower content of the banned isomeric Basic Red 118. Hence a new raw material description for the market quality has been set up (R 1200172).
The composition of Basic Brown 17 according to Ref. 4 Submission V is as follows:

Chemical Composition

Purity	
NMR Content	63 - 68 weight-%
Sum of Methylsulfate, Saccharose, Chloride and Water	< 37 weight-%
Water content	< 10 weight-%
Sulphated ash	< 12 weight-%
Heavy Metal Content	Pb < 20, Sb and Ni < 10, As and Cd < 5, Hg < 1 ppm
Purity determination by HPLC (λ =231 nm)	
Basic Brown 17	> 93 area-%
Basic Red 118	< 4.5 area-%

SCCS comment
No documentation for the above mentioned composition of "improved quality" of Basic Brown 17 was submitted.

3.1.5 Impurities / accompanying contaminants



Besides 2-nitrobenzene-1,4-diamine, Basic Red 118 and NBTRI (described in 3.1.4), several other impurities were found in B7 as shown in the chromatogram above (Basic Brown 17, Batch NDKS 1944).
Employing HPLC-MS, some impurities of Basic Brown 17 were explained by higher or lower methylated species of the starting material 7-hydroxy-N,N,N-trimethylnaphthalen-2-aminium chloride or of the main compound 8-[(4-amino-3-nitrophenyl)azo]-7-hydroxy-N,N,N-trimethylnaphthalene-2-aminium chloride.
Besides this an intermediate substance and some by-products could be found according to the mass spectra.
Taking into account the structures which could be reasonably assigned to numerous peaks detected in the HPLC were:

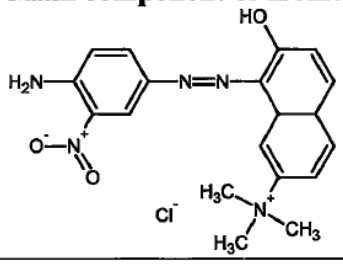
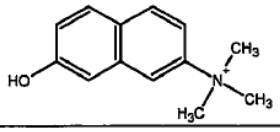
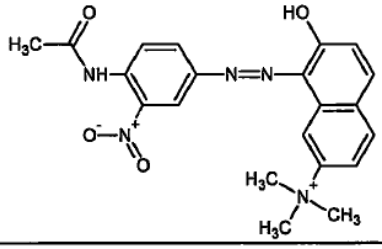
- For batch NDKS 1944, 15 of the 28 peaks accounting for 98.5 area% are identified.
- For batch 6496010, 13 of the 27 peaks accounting for 98.7 area% are identified.

Peak table with main masses detected:

RT* (min)	NDKS 1944		64960101		*m/z*
	Area	Area Frac, %	Area	Area Frac, %	
12	3	0.1	43	0.6	202.187
13	-	-	7	0.1	347
14	4	0.1	-	-	230.215
15.3	20	0.4	15	0.2	338
15.8	4	0.1	15	0.2	216.201
16.5	2	0	4	0.1	338
16.8	4	0.1	2	0	No MS-signal
17	11	0.2	18	0.2	338.323
17.3	2	0	-	-	352.1
17.5	1	0	3	0	No MS-signal
18.7	9	0.2	43	0.6	474.382
19	7	0.1	11	0.1	382
19.3	9	0.2	5	0.1	474
19.5	3	0.1	-	-	408
-	-	-	5	0.1	474
19.6	13	0.2			321.306
20.2	-	-	5	0.1	474.382
20.3	131	2.5	306	4	366.351
20.9	-	-	8	0.1	365
21.4	25	0.5	4	0	367.352
21.6	5014	93.9	7150	92.7	366.351

RT* (min)	NDKS 1944		64960101		*m/z*
	Area	Area Frac, %	Area	Area Frac, %	
22	1	0	2	0	Covered by main peak
22.1	2	0	-	-	Covered by main peak
22.2	6	0.1	9	0.1	380
-	-	-	4	0.1	518
22.8	16	0.3	5	0.1	502
23.1	9	0.2	19	0.2	379.364
23.5	5	0.1	5	0.1	502.384
24.1	9	0.2	5	0.1	502
24.6	7	0.1	9	0.1	502
25.1	16	0.3	3	0	398
25.3	4	0.1	5	0.1	502
27	3	0	-	-	521
Sum		100.1		100.1	

The interpretation of some of the m/z in the table above is described in the following table:

m/z	Interpretation
366	**Main component or Isomer (Basic Red 118)** 
	Higher methylated materials (+CH_3, -H)
380	One additional methyl-group
	Lower methylated materials (-CH_3, +H)
352	One methyl-group less
338	Two methyl-groups less
202	**Starting material** 
	Higher methylated materials (+CH_3, -H)
216	One additional methyl-group
230	Two additional methyl-groups
408	**Intermediate material** 
502	**Byproducts**

Declaration by the Applicant (Submission V)
Since Submission III (R 0500099), the quality of the product Basic Brown 17 has been improved in respect to a lower content of impurities, in particular to a lower content of the

banned isomeric Basic Red 118. Hence a new raw material description for the market quality has been set up (R 1200172).
The impurities in Basic Brown 17 according to Ref. 4 submission V are as follows:

NBTRI	< 0.8 area-%	
Higher methylated NBTRI	< 0.6 area-%	m/z (M^+) = 216, 230
Intermediate material (8-[(4-acetylamino-3-nitrophenyl)diazenyl]-7-hydroxy-*N*,*N*,*N*-trimethylnaphthalen-2-aminium chloride)	< 0.2 area-%	m/z (M^+) = 408
Byproducts	< 1.5 area-%	m/z (M^+) = 502
Byproducts	< 0.3 area-%	m/z (M^+) = 321

Impurities (quantitative)

Basic Red 118 (8-[(4-amino-2-nitrophenyl)diazenyl]-7-hydroxy-*N*,*N*,*N*-trimethylnaphthalen-2-aminium chloride	< 4.5 weight-%
NBTRI (7-Hydroxy-N,N,N-trimethylnaphthalen-2-aminium chloride)	< 1 weight-%
2-nitrobenzene-1,4-diamine	< 250 ppm

SCCS comment
No documentation for the above mentioned impurities in "improved quality" of Basic Brown 17 was submitted.

3.1.6 Solubility

Water: 16.1 g/L at 20°C, pH 5.6, EEC method A6 (Ref. 1, Submission V)
Ethanol: < 1 g/l room temperature
DMSO: 1 – 10 g/l room temperature

3.1.7 Partition coefficient (Log P_{ow})

Log Po/w: -0.1466 Method EEC A.8

3.1.8 Additional physical and chemical specifications

Organoleptic properties:	slight odour
Melting point:	200 – 202 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/

pH: /
UV_Vis spectrum (200-800 nm): λmax 216 nm and 462 nm

3.1.9 Homogeneity and Stability

Basic Brown 17 at a concentration of approximately 7.5 g/L in aqueous solution was shown to be stable for a period up to 24 h at room temperature.

Basic Brown 17 at a concentration of approximately 1 mg/ml in aqueous solution containing 0.9% sodium chloride was shown to be stable for a period up to 24 h at room temperature.

General comments to physico-chemical characterisation

The following properties do not or poorly comply with the basic requirements for proper characterisation:

- The risk assessment relates to the batches 64960101 (SAT 050019) and NDKS 1944 (SAT 000918 and SAT 040270). The quantification of the Basic Brown 17 content in these batches has been considered semi-quantitative. Both batches contain more than 25 impurities. Approximately half of the impurities have been chemically characterised and only 3 impurities were quantified. The chemical specifications of Basic Brown 17 in marketed hair dye formulations should not be significantly different from those described in section 3.1.4.
- The impurity Basic Red 118 in the two batches of Basic Brown 17 was 4.5 % (w/w) and 2.3% (w/w). According to the Cosmetic Directive, Basic Red 118 is not permitted for use in cosmetics except as an impurity in Basic Brown 17. However, the Cosmetic Directive does not mention the permitted content of Basic Red 118 as an impurity in Basic Brown 17.
- Stability of Basic Brown 17 in typical hair dye formulations was not reported.

3.2 Function and uses

Basic Brown 17 is used as a direct dye for hair colouring products. The final on head concentration of Basic Brown 17 can be up to 2.0%.

3.3 Toxicological Evaluation

(Taken from SCCS/1448/11, revision of 26/27 June 2012)

The batches have varying dye contents due to the addition of an extender. The results from the toxicity tests should be re-calculated according to dye content of the test substance.
The safety assessment relates to the batches as tested, with the indicated impurities (2-nitrobenzene-1,4-diamine 150 ppm, Basic Red 118 (2-nitro isomer of B17) 4.5%, NBTRI (7-Hydroxy-N,N,N-trimethylnaphthalene-2-aminium chloride) 0.65%.

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Taken from SCCP/0683/03

Guideline:	/
Species:	CFY rat
Group size:	4 male + 4 female
Substance:	Basic Brown 17 in 1% aqueous methylcellulose
Batch:	/
Dose:	0, 0.1, 1, 4, 8 and 16 g/kg bw in a volume of 1 to 40 ml/kg bw
Observation period:	14 days
GLP:	not in compliance
Study period:	June – July 1977

The rats (CFY strain) were treated with Basic Brown 17 with a range of dose levels from 0.1 to 16 g/kg bw. The test compound was prepared as 10 and 40% (w/v) suspensions in 1% aqueous methylcellulose. Rats dosed with the vehicle alone served as controls. All animals were observed for a period of 14 days.

Signs or reaction to treatment, observed shortly after dosing, included lethargy, piloerection, decreased respiratory rate and abnormal body carriage (hunched posture). Two male rats and one female rat from the highest dose group died. After 14 days observation, the LD50 was reported to be between 8 and 16 g/kg bw.

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

Taken from SCCP/1173/08, modified

Guideline:	OECD 404 (2002)
Species:	Albino rabbit, New Zealand White, (SPF-quality)
Group:	3 males
Substance:	B007 (Basic Brown 17)
Batch:	NDKS 1944
Purity:	96.3% (HPLC)
Dose:	0.5 g B007, moistened with water
Vehicle:	water
GLP:	in compliance
Study period:	29 June to 16 July 2004

Three rabbits were exposed to 0.5 grams of the test substance, applied onto clipped skin for 4 hours using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure.

Results
The exposure resulted in very slight erythema and/or very slight to slight oedema in the treated skin-areas of the rabbits, which had resolved within 48 hours.

Yellow-brown staining of the treated skin by the test substance was observed throughout the observation period, which did not hamper the scoring of the skin reactions. No remnants of the test substance were present on the skin.

Animal number	Mean 24 – 72 hours	
	Erythema	Oedema
35	0.3	0.0
84	0.3	0.0
82	0.3	0.3

Conclusion
The study authors concluded that the test substance was not irritant to the rabbit skin under the conditions of the study.

Ref.: 6

SCCS comment
The SCCS considers that, under the conditions of the study, the test substance showed some irritant potential to the rabbit skin.

3.3.2.2 Mucous membrane irritation / Eye irritation

Taken from SCCP/1173/08, modified

Guideline: OECD 405 (2002)
Species: Albino rabbit, New Zealand White, (SPF-quality)
Group: 3 males
Substance: B007 (Basic Brown 17)
Batch: NDKS 1944
Purity: 96.3% (HPLC)
Dose: 45 mg B007 (approximately 1 ml)
GLP: in compliance
Study period: 6 to 19 July 2004

Single samples of approximately 45 mg of B 007 (a volume of approximately 0.1 ml) were instilled into one eye of each of three rabbits. Observations were made 1, 24, 48 and 72 hours and 7 days after instillation.

Results
Instillation of the test substance resulted in effects on the iris in two animals and on the conjunctivae in all animals. Iridial irritation grade 1 was observed and had resolved within 24 or 72 hours. The irritation of the conjunctivae consisted of redness, chemosis and discharge and had completely resolved within 7 days in all animals. No corneal opacity was observed. Treatment of the eyes with 2% fluorescein, 24 hours after test substance instillation, revealed no corneal epithelial damage.
Remnants of the test substance were present in the eye 1 hour after instillation and on the outside of the eyelids 24 and 48 hours after instillation in one animal.

Animal number	Mean 24 – 72 hours			
	Corneal opacity	Iris	Conjunctivae	
			Redness	Chemosis
39	0.0	0.0	1.7	0.3
64	0.0	0.3	2.0	1.0
65	0.0	0.0	1.7	0.3

Conclusion
The study authors considered B007 not to be irritant to rabbit eyes.

Ref.: 7

SCCS comment
The SCCS considers that, under the conditions of the study, the test substance showed some irritant potential to the rabbit eye.

3.3.3 Skin sensitisation

Taken from SCCP/1173/08

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species:	Mice, CBA/CaHsdRcc (SPF)
Group:	28 females (5 test groups, 2 negative control groups) 12 females (3 positive control groups)
Substance:	B007 (Basic Brown 17)
Batch:	64960101
Purity:	77.4% (NMR)
Concentration:	0.2, 0.5, 1, 3 and 6% (w/v) B007 in ethanol/water (7/3, v/v)
Dose:	25 µl
Vehicle:	ethanol/water (7/3, v/v)
Control:	5, 10 and 25% (w/v) α-hexylcinnamaldehyde in acetone:olive oil (4:1, v/v)
Method of Analysis:	β-scintillation counter
GLP:	in compliance
Study period:	23 March – 6 July 2005

Five groups of four female mice were treated daily with the test item at concentrations of 0.2, 0.5, 1, 3 and 6% (w/v) in ethanol/water (7/3, v/v) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. 6% was the highest technically applicable concentration in the vehicle. Two control groups of four mice each were treated with the vehicle (ethanol/water (7/3, v/v)) only. Three positive control groups of four mice each were treated with 5, 10 and 25% (w/v) α-hexylcinnamaldehyde in acetone:olive oil (4:1, v/v) in a separate study.

Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a ß-scintillation counter.

No clinical signs were observed in any animals of the control groups. On the third application day, a slight erythema was observed at both dosing sites in all mice of the 3%-group. From the second application day, a moderate or slight erythema was observed at both dosing sites in all mice of 6%-group, persisting for the remainder of the in-life phase of the study.

The results obtained (Stimulation Index (S.I.)) are reported in the following table.

Concentration (%)	S.I.
Test item	
0.2	1.0
0.5	1.0
1	1.3
3	0.9
6	1.3
α-hexylcinnamaldehyde	
5	2.4
10	3.6
25	11.2

No test item related findings, such as significant body weight loss or local/systemic findings were observed up to the concentration of 1 %. At the higher concentrations tested, i.e. 3 and 6%, some test item related signs, such as slight to moderate ear erythema, were observed at the local dosing sites but no clear change of dpm/LN was caused by this local irritant effect. All treated animals survived the scheduled study period.

Conclusion
The study authors concluded that the test item did not show an allergenic potential when tested up to the concentration of 6 % (w/v) in ethanol/water (7/3, v/v).

Ref.: 8

SCCS comment
The vehicle used is not a recommended vehicle for LLNA. The maximum test concentration is too low. A conclusion on sensitisation cannot be drawn.

3.3.4 Dermal / percutaneous absorption

New study, submission IV, 2010

Guideline:	OECD Guideline 428
Test substance:	Basic Brown 17
Batch:	64960101
Formulation batch:	FGS09MK0003/01
Purity:	77.4%
Tissue:	dermatomed pig skin from the back region (2 females, 2 males) (780-800 µm)
Skin integrity:	skin electrical resistance measurement (>4 kΩ)
Method:	static diffusion cell 3.14 cm²
Receptor fluid:	sodium chloride, 0.9% w/v
Formulation tested:	typical hair dye formulation (2% w/w)
Dose formulation applied:	21.18 mg/cm² of the formulation equivalent to 20 mg/cm² of test substance
Concentration ingredient:	1.58% active ingredient (2% in formulation)
Solubility receptor fluid:	water: 10 – 100 g/l, room temperature
Replicate cells:	10 (2 of 12 were rejected low mass balance)
Duration of the contact:	30 min
Duration of the diffusion:	24 hours (sampling time: 0.5, 1, 2, 4, 6, and 24 h)
Analytical method:	LC-MS/MS
GLP:	in compliance
Study period:	September 2009- February 2010

The dermal bioavailability of Basic Brown 17 has been studied, following topical application of a typical hair dye formulation (2.0%, w/w) to excised dermatomed pig skin.
Previously frozen dermatomed pig skin from 2 males and 2 females was mounted into static diffusion cells (12 replicates in total) containing receptor fluid (sodium chloride, 0.9% w/v) in the receptor chamber. The skin surface temperature was maintained at ca 32°C throughout the experiment. An electrical resistance barrier integrity test was performed and any pig skin sample exhibiting a resistance <4 kΩ was excluded from subsequent dermal bioavailability measurements.
20 mg/cm2 of the hair dye formulation was applied to dermatomed pig skin mounted into static diffusion cells in vitro.
Dermal bioavailability was assessed by collecting receptor fluid aliquots at 0.5, 1, 2, 4, 6 and 24 h post dose. At 30 min post dose exposure was terminated by washing the skin surface with a dilute shampoo solution and water. The skin surface was dried with tissue paper (tissue swabs). At 24 h post dose, the washing procedure was repeated. The skin was then removed from the static diffusion cells, dried and the stratum corneum was removed with 20 successive tape strips. The remaining skin was divided into exposed and unexposed skin.
The receptor fluid in the receptor chamber was removed into a bulk receptor fluid vial and stored at ambient temperature. All samples were analysed by LC-MS/MS.

Results
The dermal bioavailability of Basic Brown 17 following topical application of the compound in hair dye formulation to pig skin in vitro was 0.48% (1.62 µg/cm2) of the applied dose. The majority of the dose was removed by washing the skin. The mass balance was complete (102.47%).

Table 1 Distribution of B007 (% Applied Dose) at 24 h Post Dose Following 30 Min Topical Application of B007 in Hair Dye Formulation to Dermatomed Pig Skin (Mean + SD, n = 10)

	Cell Number and Animal Number													
	Cell 1 07M	*Cell 2 07M*	Cell 3 07M	Cell 4 41F	Cell 5 41F	Cell 6 41F	Cell 7 37F	Cell 8 37F	Cell 9 37F	Cell 10 04M	*Cell 11 04M*	Cell 12 04M	Mean	SD
Skin Wash 0.5 h	28.72	*56.21*	33.41	65.97	32.85	63.63	22.24	51.24	13.80	58.37	*10.42*	89.16	45.94	23.53
Tissue Swab 0.5 h	62.57	*22.52*	68.30	32.85	65.69	36.24	68.30	47.65	75.08	40.88	*58.39*	11.00	50.86	20.52
Dislodgeable Dose 0.5 h	91.28	*78.74*	101.71	98.82	98.54	99.86	90.54	98.89	88.87	99.25	*68.81*	100.16	96.79	4.65
Skin Wash 24 h	3.45	*0.67*	2.50	0.97	2.45	0.83	5.60	1.22	9.48	0.98	*3.27*	0.30	2.78	2.84
Tissue Swab 24 h	2.32	*0.56*	2.91	0.66	1.65	0.46	1.60	0.66	3.50	0.35	*1.90*	0.10	1.42	1.18
Donor Chamber Wash	0.46	*0.27*	0.47	0.30	0.46	0.46	0.28	0.83	0.60	0.00	*1.02*	0.00	0.39	0.25
Total Dislodgeable Dose	97.52	*80.24*	107.59	100.75	103.10	101.61	98.02	101.60	102.46	100.58	*75.00*	100.57	101.38	2.80
Unexposed Skin	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Total Unabsorbed	97.52	*80.24*	107.59	100.75	103.10	101.61	98.02	101.60	102.46	100.58	*75.00*	100.57	101.38	2.80
Stratum Corneum 1	0.04	*0.07*	0.07	0.01	0.04	0.03	0.12	0.17	0.27	0.13	*0.10*	0.05	0.09	0.08
Stratum Corneum 2	0.10	*0.29*	0.19	0.02	0.03	0.03	0.04	0.17	0.14	0.09	*0.16*	0.03	0.08	0.06
Stratum Corneum 3	0.06	*0.30*	0.06	0.00	0.02	0.03	0.05	0.10	0.11	0.11	*0.05*	0.02	0.06	0.04
Stratum Corneum 4	0.06	*0.11*	0.02	0.03	0.05	0.02	0.02	0.05	0.07	0.08	*0.02*	0.04	0.04	0.02
Stratum Corneum 5	0.09	*0.11*	0.16	0.01	0.01	0.00	0.05	0.07	0.05	0.04	*0.03*	0.02	0.05	0.05
Stratum Corneum 6-10	0.10	*0.18*	0.53	0.06	0.06	0.11	0.12	0.18	0.25	0.16	*0.09*	0.05	0.16	0.14
Stratum Corneum 11-20	0.07	*0.21*	0.34	0.07	0.09	0.12	0.12	N.S.	0.18	0.13	*0.27*	0.09	0.13	0.08
Dermal Adsorption	0.53	*1.26*	1.36	0.20	0.29	0.34	0.51	0.74	1.06	0.73	*0.73*	0.31	0.61	0.37
Dermal Absorption (Exposed Skin)	0.67	*0.84*	0.82	0.67	0.24	0.70	0.30	0.33	0.33	0.58	*0.61*	0.20	0.48	0.22
Receptor Fluid	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Receptor Chamber Wash	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Percutaneous Penetration	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Dermal Bioavailability	0.67	*0.84*	0.82	0.67	0.24	0.70	0.30	0.33	0.33	0.58	*0.61*	0.20	0.48	0.22
Mass Balance	98.72	*82.34*	109.77	101.61	103.64	102.65	98.83	102.67	103.86	101.88	*76.33*	101.08	102.47	3.11

Zero values are <LLOQ N.S. = no sample *Cells 2 and 11 rejected form Mean and SD due to low mass balance (<90%)*

Table 2 **Distribution of B007 (µg/cm²) at 24 h Post Dose Following 30 min Topical Application of B007 in Hair Dye Formulation to Dermatomed Pig Skin (Mean + SD, n = 10)**

	Cell Number and Animal Number													
	Cell 1 07M	*Cell 2 07M*	Cell 3 07M	Cell 4 41F	Cell 5 41F	Cell 6 41F	Cell 7 37F	Cell 8 37F	Cell 9 37F	Cell 10 04M	*Cell 11 04M*	Cell 12 04M	Mean	SD
Skin Wash 0.5 h	96.19	*188.30*	111.91	220.99	110.03	213.14	74.50	171.64	46.21	195.53	*34.89*	298.64	153.88	78.83
Tissue Swab 0.5 h	209.57	*75.45*	228.78	110.03	220.05	121.38	228.78	159.62	251.49	136.92	*195.60*	36.85	170.35	68.73
Dislodgeable Dose 0.5 h	305.77	*263.75*	340.69	331.02	330.08	334.51	303.29	331.26	297.70	332.45	*230.49*	335.49	324.23	15.58
Skin Wash 24 h	11.57	*2.25*	8.36	3.24	8.20	2.78	18.77	4.09	31.75	3.27	*10.94*	1.02	9.30	9.50
Tissue Swab 24 h	7.77	*1.87*	9.76	2.20	5.52	1.54	5.34	2.20	11.74	1.17	*6.37*	0.35	4.76	3.94
Donor Chamber Wash	1.55	*0.91*	1.57	1.01	1.55	1.54	0.94	2.77	2.03	0.00	*3.41*	0.00	1.30	0.85
Total Dislodgeable Dose	326.66	*268.77*	360.39	337.47	345.35	340.37	328.34	340.32	343.21	336.89	*251.22*	336.86	339.59	9.39
Unexposed Skin	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Total Unabsorbed	326.66	*268.77*	360.39	337.47	345.35	340.37	328.34	340.32	343.21	336.89	*251.22*	336.86	339.59	9.39
Stratum Corneum 1	0.15	*0.23*	0.22	0.05	0.14	0.12	0.39	0.56	0.89	0.42	*0.34*	0.18	0.31	0.26
Stratum Corneum 2	0.32	*0.98*	0.63	0.05	0.11	0.09	0.12	0.57	0.48	0.29	*0.54*	0.10	0.28	0.22
Stratum Corneum 3	0.19	*1.00*	0.20	0.00	0.05	0.11	0.17	0.35	0.37	0.36	*0.16*	0.06	0.19	0.14
Stratum Corneum 4	0.21	*0.36*	0.05	0.09	0.16	0.05	0.06	0.15	0.24	0.25	*0.08*	0.13	0.14	0.08
Stratum Corneum 5	0.32	*0.36*	0.54	0.04	0.04	0.00	0.18	0.24	0.15	0.14	*0.09*	0.06	0.17	0.16
Stratum Corneum 6-10	0.35	*0.59*	1.77	0.19	0.19	0.37	0.39	0.60	0.84	0.54	*0.30*	0.20	0.54	0.48
Stratum Corneum 11-20	0.24	*0.70*	1.14	0.24	0.29	0.41	0.39	N.S.	0.59	0.45	*0.91*	0.30	0.45	0.28
Dermal Adsorption	1.77	*4.22*	4.56	0.67	0.99	1.14	1.71	2.47	3.56	2.45	*2.43*	1.04	2.03	1.25
Dermal Absorption (Exposed Skin)	2.25	*2.80*	2.75	2.23	0.81	2.33	1.02	1.12	1.11	1.93	*2.04*	0.67	1.62	0.75
Receptor Fluid	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Receptor Chamber Wash	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Percutaneous Penetration	0.00	*0.00*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.00*	0.00	0.00	0.00
Dermal Bioavailability	2.25	*2.80*	2.75	2.23	0.81	2.33	1.02	1.12	1.11	1.93	*2.04*	0.67	1.62	0.75
Mass Balance	330.67	*275.80*	367.70	340.37	347.14	343.83	331.06	343.90	347.88	341.28	*255.69*	338.57	343.24	10.41

Zero values are <LLOQ N.S. = no sample *Cells 2 and 11 rejected form Mean and SD due to low mass balance (<90%)*

Ref.: 16 (subm. IV)

SCCS comment
The experiment is well performed. Under non-oxidative conditions, the mean + 1SD (1.62 + 0.75) = 2.37 µg/cm^2 should be used to calculate MoS of Basic Brown 17.

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) toxicity (oral)

Taken from SCCP/1173/08

Guideline:	OECD 408
Species/strain:	Rat, Wistar Hannover (HsdBrlHan:Wist)
Group size:	20 (10 per sex) + recovery group (control/high dose 5 per sex)
Test substance:	B 007
Batch:	64960101
Purity:	77.4% (NMR)
Dose:	0, 60, 120 and 180 mg/kg bw/day
Vehicle:	distilled water
Route:	gavage at a dose volume of 10 ml/kg bw
Exposure:	13 weeks + 4 week recovery control/high dose groups
GLP:	in compliance
Date:	12 Dec 2005

During the study, clinical signs, death, body weight and food consumption were recorded. The recovery groups were additionally examined during the 4-week treatment-free period.

At the end of the study, the animals were killed and routine pathological investigations performed.

Results
Two control males and 2 males and 1 female receiving 180 mg/kg bw/day B 007 were found dead during the treatment period. A control male died of accidental trauma on day 18, while a recovery group control male died on day 34. The high dose female died on day 21, the two high dose males on days 57 and 87 of dosing.
Body weight and food consumption were unaffected by treatment. No ophthalmological changes occurred during treatment.
During the daily pre- and post-dose observations, no clinical signs were detected. At the weekly clinical signs and functional observation tests, no significant differences between control and test substance-treated animals were seen.
There were no changes in motor activity in animals receiving the test substance compared with controls. Tail pinch response was slightly increased in high dose animals at the end of the treatment period but not at the end of recovery.
There were no significant haematological changes noted during the study. Two mid-dose females and six high dose (three females and three males) showed moderate to marked alteration in aspartate aminotransferase. Since only one of these high dose female also had raised alanine aminotransferase, liver injury was excluded. In the high dose group, significantly raised gamma-glutamyl transferase, cholesterol, triglycerides, glucose levels and lowered sodium were observed. At the end of the recovery phase, these parameters were similar to the control values.

Some of high dose animals showed bilirubinuria, but as no hyperbilirubinaemia was observed, it was thought that this could be due to the presence of the test substance and/or its metabolites in the urine.

Post mortem results unscheduled deaths
The post mortem examinations of both the control males and the high dose female, which were found dead, showed incomplete collapse in the lungs. Both the lungs and thymus had a dark/red colour. In the other high dose male deaths, the lungs, thymus, spleen and thyroid had also dark/red colour. In one, other changes noted were firm consistency of the heart, irregular surface and swelling of the liver and oedematous consistency of the prostate.

Post mortem results treatment groups
The high dose rats showed dark coloration in the brain, heart, kidneys, ovaries, skeletal muscle, spleen and thyroid with the females more effected than males. In the mid dose, both sexes showed dark coloration of the spleen and thyroid. In addition, some females had dark coloration in the heart and skeletal muscle.

Post mortem results recovery group
Dark coloration was still present after 4 weeks of recovery in the brain, heart, skeletal muscle, spleen and thyroid of treated males and females, when compared with the controls.

Histopathology unscheduled deaths
Moderate to marked myocarditis, centrilobular hepatocytic necrosis with instances of acute inflammation and moderate to marked lymphoid depletion of the thymus were described in both males dosed at 180 mg/kg bw/day and were seen as the possible cause of death.
The most important changes observed in the treated female were pulmonary congestion, lymphocytolysis of the mesenteric lymph nodes and thymus and lymphoid depletion of the spleen. Accidental damage was considered the cause of moderate haemorrhage in the lungs and thymus in 1 of the 2 control males.

Histopathology treatment group (90 day)
Yellow/brown pigmentation was the most relevant treatment-related change observed in the heart, kidneys, liver, spleen, thyroids, Peyer's patches (ileum) and skeletal muscle of animals, both sexes, receiving ≥ 120 mg/kg bw/day, when compared with controls.
Yellow/brown pigmented macrophages were also seen in the lungs of females receiving ≥ 120 mg/kg bw/day. Treated males were similar to the controls.
In addition, males and females dosed at 180 mg/kg bw/day showed yellow/brown pigmentation in the adrenals, ovaries, uterus, mesenteric/cervical lymph nodes and thymus.
An increased incidence of extramedullary haemopoiesis in the spleen in all treated groups was described.
The remaining lesions, including the statistically significant increased incidence of inflammatory cell foci described in the heart of males dosed at 60 mg/kg bw/day were considered either incidental in origin or an expression of spontaneous pathology, commonly seen in this species under the experimental conditions used.

Histopathology recovery group
Yellow/brown pigmentation was still seen in the adrenals, heart, kidneys, liver, ovaries, spleen, thyroid, skeletal muscle and mesenteric/cervical lymph nodes in the high dose group.
Absolute and relative thyroid weights were lower than controls in females dosed at 180 mg/kg bw/day at the end of the recovery period; in the same animals the relative liver weights were higher than controls.

Changes in clinical chemistry parameters that could be correlated with the histopathological changes detected in the heart and skeletal muscle were a moderate to marked increase in aspartate aminotransferase detected in some animals dosed at 120 and 180 mg/kg bw/day at the end of the treatment period.

Conclusion
On the basis of this study, the low dose level of 60 mg/kg bw/day may be considered the No Observed Adverse Effect Level (NOAEL). As the dye content of the test substance was only 77.4%, the NOAEL is recalculated to 46 mg/kg bw/day (60 x 0.774).

Ref.: 12 (2005 submission)/
14 (2010 submission VI)

3.3.5.3 Chronic (> 12 months) toxicity

No data submitted

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537.
Replicates:	triplicates in only one experiment both in the presence and absence of S9- mix.
Test substance:	B 007
Solvent:	deionised water
Batch:	64960101
Purity:	77.4% (NMR)
Concentrations:	3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix
GLP:	in compliance
Study period:	January - April 2007

B 007 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 in a preliminary toxicity test 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 spontaneous revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in the strains used, the pre-experiment is reported as part of the main experiment. Both the pre and main experiment were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of B 007 was observed from 2500 µg/plate and above in the pre-experiment and from 333 µg/plate and above in the overlay agar of the main experiment. The undissolved particles of B 007 had no influence on the data recording. Both without and with S9-mix toxic effects were observed at 333 µg/plate for TA100 and TA102 and at 2500 µg/plate for TA98 and TA1537. Toxic effects were not observed for TA1535.

B 007 treatment resulted in a biologically relevant and dose dependent increase in the number of revertant colonies in strains TA98 and TA 1537. At the two highest concentrations, the number of revertant colonies was reduced due to overlapping toxic effects.

Conclusion
Under the experimental conditions used, B 007 was mutagenic in this gene mutation tests in bacteria.

Ref.: 9

SCCS comment
Since a positive result was obtained in the first experiment, a second experiment was not performed.

In Vitro **Mouse Lymphoma assay (*tk* locus*)***

Guideline:	OECD 476 (1998)	
Cells:	L5178Y Mouse lymphoma cells	
Replicates:	two parallel cultures in 2 independent experiments	
Test substance:	B 007	
Solvent:	deionised water	
Batch no.:	64960101	
Purity:	77.4% (NMR)	
Concentrations:	Experiment I:	8.1, 16.3, 32.5, 65.0 and 97.5 µg/ml without S9-mix 16.3, 32.5, 65.0, 130.0 and 195.0 µg/ml with S9-mix
	Experiment II:	8.0, 16.0, 32.9, 64.0, 128.0 and 192.0 µg/ml without S9-mix
Treatment	Experiment I:	4 h treatment without and with S9-mix; expression period 72 h and selection period of 10-15 days
	Experiment II:	24 h treatment without S9-mix; expression period 48 h and selection period of 10-15 days
GLP:	In compliance	
Study period:	January - June 2005	

B 007 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. In the main test, cells were treated for 4 h or 24 h (without S9-mix in experiment II only) followed by an expression period of 72 or 48 h 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 percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In experiment I, precipitation was noted at 97.5 and 130.0 µg/ml without S9-mix and at 195.0 and 260.0 µg/ml with S9-mix; in experiment II precipitation occurred at 128.0 and 192.0 µg/ml, respectively.
The appropriate level of toxicity (10-20% survival after the highest dose) was not reached in the experiment with S9-mix pointing to insufficient exposure of the cells.
Both in experiment I and II no biological relevant and concentration dependent increase in the number mutant colonies was observed independent of the presence or absence of S9-mix.

Conclusion

Under the experimental conditions used, B 007 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref.: 10

SCCS comment
The appropriate level of toxicity (10-20% survival after the highest concentration) was not reached in the experiment with S9-mix which may point to insufficient exposure of the cells. Historical control data were only reported for the total number of mutant colonies; historical data for "small" and "large" colonies were not available.

Gene mutation test in mammalian cells (*hprt* locus*)*

Guideline:	OECD 476 (1997)	
Cells:	L5178Y mouse lymphoma cells	
Replicates:	duplicate cultures in two independent tests	
Test substance:	B007	
Batch:	64960101	
Solvent:	deionised water	
Purity:	98.7 area% by HPLC	
Concentrations:	experiment I:	3.1, 6.2, 12.4, 24.8, 49.6 and 99.2 µg/ml without S9-mix 6.2, 12.4, 24.8, 49.6, 99.2 and 198.4 µg/ml with S9-mix
	experiment II	3.1, 6.2, 12.4, 24.8, 37.2 and 49.6 µg/ml without S9-mix 3.1, 6.2, 12.4, 24.8, 49.6 and 99.2 µg/ml with S9-mix
Treatment	experiment I:	3 h both without and with S9-mix; expression period 48 h and a selection period of 10-15 days. 24 h without S9-mix; expression period 48 h and a selection period of 10-15 days. 3 h with S9-mix; expression period 48 h and a selection period of 10-15 days.
GLP:	in compliance	
Study period:	22 August 2012 – 22 October 2012	

B007 was assayed for gene mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. A pre-test on toxicity, using identical conditions as in the main test, was performed to determine the concentration range for the mutagenicity experiments using several concentrations between 12.7 and 3256 µg/ml. For the 4 h treatment period serum concentration was reduced from 15% to 3%. Toxicity was measured as percentage suspension growth relative to the suspension growth of the solvent control cultures.
In the main tests, cells were treated for 4h without and with S9-mix or for 24h without S9-mix followed by an expression period of 48h to fix the DNA damage into stable *hprt* mutations. A serum concentration of 3% was used. Toxicity was measured as percentage survival of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In both experiments without and with metabolic activation, precipitation was observed at 49.6 µg/ml and above. Exclusively, in experiment I without metabolic activation, the required relative survival of 10-20% as compared to the untreated control was reached.
A biological relevant increase of the mutant frequency was not observed in both experiments with and without metabolic activation. An isolated increase exceeding the limit

of three times the mutant frequency of the untreated control was observed in one culture of experiment II with metabolic activation at the highest concentration (99.2 µg/ml). However, precipitation occurred at this concentration and the increase was not concentration dependent. Therefore, this isolated effect was considered not biologically relevant.

Conclusion
Under the test conditions used B007 did not induce gene mutations at the *hprt* locus in mouse lymphoma cells and is not considered mutagenic in this assay.

Ref.: 6 (subm. V)

SCCS comment
The appropriate level of toxicity (10-20% survival after the highest concentration) was not reached in experiment II without S9-mix and in both experiments with S9-mix which may point to insufficient exposure of the cells.

***In vitro* micronucleus test**

Guideline:	draft OECD 487 and OECD 473 (*in vitro* chromosomal aberration test)	
Cells:	Chinese hamster V79 cells	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	B 007	
Solvent:	deionised water	
Batch:	64960101	
Purity:	77.4% (NMR)	
Concentrations:	experiment IA:	128.1, 256.3, 2050.0 and 4100.0 µg/ml without S9-mix
	experiment IA:	128.1, 256.3, 1025.0 and 2050.0 µg/ml with S9-mix
	experiment IB:	31.3, 62.5, and 125.0 without S9-mix
	experiment IIA:	128.1, 256.3 and 512.5 µg/ml without S9-mix
	experiment IIA:	128.1, 256.3, 512.5 and 1025.0 µg/ml with S9-mix
	experiment IIB:	100.0, 150.0, 200.0, 250.0, 300.0 and 350.0 µg/ml without S9-mix
	experiment IIB:	100.0, 200.0, 400.0, 600.0 and 800.0 µg/ml with S9-mix
Treatment	experiment IA and IB:	4 h treatment; harvest time 24 hours after the beginning of treatment, without S9-mix
	experiment IA:	4 h treatment; harvest time 24 hours after the beginning of treatment, with S9-mix
	experiment IIA and IIB:	20 h treatment; harvest time 24 hours after the beginning of treatment without S9-mix
	experiment IIA and IIB:	4 h treatment; harvest time 48 hours after the beginning of treatment with S9-mix
GLP:	In compliance	
Study period:	February - September 2005	

B 007 has been 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 007, the solubility during exposure and thus the test concentrations for the main micronucleus test. The highest concentration should produce clear toxicity with reduced cell growth. Considering the toxicity data of the pre-test and the occurrence of precipitation of B 007, 4100 µg/ml (≈ 10 mM the prescribed maximum concentration) was chosen as top concentration in experiment IA. To corroborate the data of this experiment in the absence of S9-mix, a confirmatory experiment (experiment IB) was performed with a top concentration of 500 µg/ml. Concentration selection in experiment IIA was influenced by Basic Brown 17 toxicity and precipitation observed in

experiment I. Due to the steep concentration toxicity curve, a repeat experiment (experiment IIB) was performed with narrower dilution steps to prove if genotoxicity observed at highly toxic concentrations far below the 40% of control level was an artificial finding.
The treatment period in the main test was 4 h in experiment I (without and with S9-mix) and in experiment II (with S9-mix) or 20 h in experiment II (without S9-mix). Harvest time was 24 h or 48 h (experiment II with S9-mix only) after the beginning of culture. For assessment of cytotoxicity a XTT test was additionally carried out in parallel to the main micronucleus test. Negative and positive controls were in accordance with the draft guideline.

Results
In all experiments clear toxic effects indicated by reduced cell numbers below 40% of control were observed at least at the highest concentrations scored after treatment with B 007 except in experiment IB in the absence of S9-mix.
In experiment IA, in the absence of S9-mix, a statistically significant but non-concentration-related increase in the rate of micronucleated cells was observed at the lowest and highest concentration. The values of highest concentration were at the laboratory's control data range (0.0 – 1.8% micronucleated cells). Concerning the lowest concentration, in the confirmatory experiment IB this finding was not confirmed. Consequently, the positive finding was considered not biologically relevant. In experiment IA, in the presence of S9-mix no biologically relevant increase in the percentage of micronucleated cells was observed after treatment with the test item.
In experiment IIA, in the absence and the presence of S9-mix, a statistically significant increase in the number of micronucleated cells exceeding the range of the historical control data was observed at the highest concentrations (512.5 and 1025 µg/ml, respectively). These concentrations were strongly cytotoxic indicated by cell numbers of 7.9% and 12.9% of control, respectively.
Due to the steep concentration-toxicity curve a repeat experiment, designated experiment IIB, was performed with narrower dilution steps to prove if the genotoxicity observed could have been an artefact induced by general test item toxicity. In the absence of S9-mix, at a cytotoxic level of about 40% of control the number of micronucleated cells (2.05% and 2.00%) slightly exceeded the historical control data range (0.0 – 1.8% micronucleated cells). Therefore, the test item was regarded as non-genotoxic in the absence of metabolic activation.
In the presence of S9-mix, at cytotoxic test item levels and associated with precipitation from concentrations equal or exceeding 200 µg/ml, the number of micronucleated cells (2.68% and 2.33%) slightly exceeded the range of the historical control data (0.0 – 1.8% micronucleated cells). Due to the high value of the respective solvent control (1.80% micronucleated cells), these two slight increases have to be regarded as biologically irrelevant.
The observations of experiment IIA in the absence and the presence of metabolic activation were not confirmed in the repeat experiment IIB with narrower dilution steps. Therefore, it has to be considered that the findings in both parts of experiment IIA were artefacts induced by general test item toxicity.

Conclusion
Under the experimental conditions used, the test substance did not induce an increase in micronucleated cells and, consequently, is not genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref.:11

Single cell gel electrophoresis assay (COMET assay) in reconstructed human skin

Guideline: /
Tissue: Phenion® Full-Thickness Skin

Replicates:	3 tissues/concentration in 3 independent experiments
Tissue batches:	13-05, 13-06 and 13-08
Test substance:	B007
Batch:	64960101
Purity:	98.7 area% (by HPLC, 77.4 weight% (by ^{1}H-NMR))
Solvent:	70% ethanol
Concentrations:	2000, 4000, 6000 and 8000 µg/ml (corresponding to 33, 65, 97 and 130 µg/cm^2)
Treatment:	3h treatment
GLP:	/
Study period:	August 2012 – April 2013

B007 has been investigated for induction of DNA damage in reconstructed human skin tissue using the Comet assay. A reconstructed three dimensional skin tissue made of non-transformed human keratinocytes (epidermis) and fibroblasts (dermis) was used. The tissue allows realistic exposure conditions for B007 by topical application and the consideration of the species- and organ-specific metabolism of the compound. Tissues were exposed to quantities of 25 µl on 16 µl/cm^2. The tissues had a surface of 1.54 cm^2.
The actual concentrations used in the main experiment were based on the results of a concentration range-finder experiment, with concentrations ranging up to 100000 µg/ml (1600 µg/cm^2). As the concentration range finding experiment met the acceptability criteria of the comet assay it was taken as the first main experiment.
In the main experiments, 2000, 4000, 6000 and 8000 µg/ml (corresponding to 33, 65, 97 and 130 µg/cm^2) were investigated in each experiment. All concentration groups comprised 3 tissues. Three independent experiments were performed to increase the statistical power of the biological deviation in this *in vitro* culture system. Three slides were prepared from both the dermis and epidermis of each skin tissue: at least 2 of the 3 slides were analysed.
Tissues were treated for 3 h. After treatment, the tissues were incubated with thermosolyn for 2h to allow the degradation of the basal membrane between the dermis and the epidermis and the dermis was peeled off from the epidermis. From both the dermis and epidermis a cell suspension was made mechanically.
Electrophoresis was performed for 30 min at 1 V/cm. For the evaluation of Comets the % tail DNA (= tail intensity) was used as assessment parameter. 50 cells per slide, 3 slides per tissue compartment (dermis and epidermis) and 4 tissues per concentration were scored. Cytotoxicity was determined by measuring the intracellular ATP concentration and the activity of the adenylate kinase released into the culture medium. Appropriate negative and positive controls were included.

Results
8000 µg/ml (130 µg/cm^2) of B007 in 70% ethanol was shown to be the highest concentration applicable to the skin tissue due to its limited solubility and it was the lowest concentration that gave rise to precipitation.
The values obtained for both cytotoxicity parameters were fully acceptable according the criteria defined. Only the values for adenylate kinase at the highest and lowest B007 concentrations in the third experiment were slightly above the cytotoxicity limit of 200%. However, as B007 did not induce an increase in % tail DNA in this experiment the increased cytotoxicity was considered not to compromise the validity of this third experiment.
In the first experiment in fibroblasts only (dermis) a statistically significant increase in tail intensity was observed at the highest concentration tested. No increases were observed at the other concentrations. In the second and third experiment, a biologically relevant increase in % tail DNA in either keratinocytes or fibroblast was not observed.
Since the isolated increase of the first experiment was not reproduced in the two other experiments, this isolated effect was considered not biologically relevant.

Ref.: 7 (subm. V)

Conclusion

Under the experimental conditions used, B007 was not genotoxic in this *in vitro* alkaline Comet assay in reconstructed human skin tissue.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

No data submitted

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

Taken from SCCP/1173/08

Guideline:	OECD 414
Species/strain:	Rat, Wistar HsdBrlHan: female
Group size:	25
Test substance:	B-007
Batch:	64960101
Purity:	77.4% (NMR)
Dose:	0, 60, 120, 240 mg/kg bw/d in distilled water
Route:	Gavage, 10 ml/kg bw
Exposure:	Gestation Day (GD) 5-19
GLP:	in compliance
Date:	7 November 2005

Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed in situ were considered to be at GD 0 and assigned to individual housing. Animals were also examined for clinical observations, general appearance, abortions, premature deliveries and death daily. Food consumption and body weight were recorded on GD 0, 5, 9, 12, 15 and 20. On GD 20, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section.

Results

Maternal
During the study, there was one unscheduled high dose death on GD 11. Macroscopic examination showed enlarged adrenals, abnormal swollen intestinal tract content, whereas liver and spleen had a dark colour.
A total of nine females were not pregnant at termination: two in the control group, five in the low dose group and one each in the mid- and high dose groups. Unilateral implantation was present in one high dose female.
The number of females with live foetuses on GD 20 was 23 in the controls, 20 in the low dose group, 24 in the mid-dose group and 23 in the high dose.
Scabs and hair loss were the principal clinical signs observed in the treated females during the treatment period, but also occurred occasionally in the control group.
Abrasion and aggressive behaviour were noted in two different high dose females on GD 19 and 20, respectively. Dyspnoea was recorded in one low dose female on GD 7. No other

signs of reaction to treatment were recorded at the daily pre- and post-dose observations. Light to dark brown staining was observed on the cage floor in the high dose group, considered to be caused by the test substance, probably eliminated in the urine.
When compared with the controls, statistically significant reductions in body weight gain (GD 9) and food consumption (GD 9 and 12) were noted in the high dose group. Aligned with this, was a statistically significant lower terminal body weight and consequently absolute weight gain in the high dose group compared with the control. Gravid uterus weight was not affected by treatment. At *post mortem*, the spleen was dark and occasionally swollen in the high dose females. This was considered related to the colour of the test item. Other findings were not dose related and considered to be incidental or spontaneous.
At all dose levels, the litter means for *corpora lutea*, implantations, litter sizes, live foetuses, early and late resorptions, foetal body weights, percent resorbed conceptuses, and percent live male foetuses were similar to the controls. No dams had litters of only resorbed conceptuses, and there were no dead foetuses.

Foetal parameters
13 small foetuses were found (control: 4, low dose: 3, mid-dose: 5 and high dose: 1). In one mid-dose foetus, brain ventricles were enlarged. This was considered incidental. There were no other dose-dependent, significant differences in the litter or foetal incidences of any gross external, soft tissue or skeletal alterations.

Conclusion
The maternal No Observed Adverse Effect Level (NOAEL) was considered to be 120 mg/kg bw/day. The foetal No Observed Adverse Effect Level (NOAEL) was higher than 240 mg/kg bw/day. As the dye content of the test substance was only 77.4%, the NOAELs were recalculated to 93 mg/kg bw/day (maternal) and 186 mg/kg bw/day (foetal).

Ref.: 13

3.3.9 Toxicokinetics

No data submitted.

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

No data submitted.

3.3.10.2 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

Basic Brown 17

(Non-oxidative conditions)
(2.0% formulation, on head concentration 2,0%)

Absorption through the skin	**A**	**= 2.37 µg/cm²**
Skin Area surface	**SAS**	**= 580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**= 1.38 mg**
Typical body weight of human		**= 60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**= 0.023 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**= 46 mg/kg bw/d**
Bioavailability 50%*		**= 23 mg/kg bw/d**

Margin of Safety	**adjusted NOAEL/SED**	**= 1000**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Physico-chemical properties

Basic Brown 17 is used as a direct dye for hair colouring products. It is used without mixing with an oxidising agent (*e.g.* hydrogen peroxide). The final on head concentration of Basic Brown 17 can be up to 2.0%.

The batches have varying dye contents due to the addition of an extender. The results from the toxicity tests should to be re-calculated according to dye content of the test substance.

The risk assessment relates to the batches 64960101 (SAT 050019) and NDKS 1944 (SAT 000918 and SAT 040270). The content of Basic Brown 17 in these batches has been considered semi-quantitative. Both batches contain more than 25 impurities. Approximately half of the impurities have been chemically characterised and only 3 impurities were quantified. The chemical specifications of Basic Brown 17 in marketed hair dye formulations should not be significantly different from those described in section 3.1.4.

The impurity Basic Red 118 in the two batches of Basic Brown 17 was 4.5 % (w/w) and 2.3% (HPLC peak area). According to the Cosmetic Directive, Basic Red 118 is not permitted for use in cosmetics except as an impurity in Basic Brown 17. However, the Cosmetic Directive does not mention the permitted content of Basic Red 118 impurity in Basic Brown 17.

Stability of Basic Brown 17 in in typical hair dye formulations was not reported.

General toxicity

After 14 days observation, the LD_{50} was reported to be between 8 and 16 g/kg bw.

On the basis of a 90-day study, the No Observed Adverse Effect Level (NOAEL) was 60 mg/kg bw/day. As the dye content of the test substance was only 77.4%, the NOAEL is recalculated to 46 mg/kg bw/day (60 x 0.774). This NOAEL should be used for the calculation of MoS.

The NOAEL for maternal toxicity was considered to be 93 mg/kg bw/day. The NOAEL for foetal toxicity was higher than 186 mg/kg bw/day (both values also corrected for dye content).

No study on reproductive toxicity was provided.

Irritation / sensitisation
Under the conditions of the study, the test substance showed some irritant potential to the rabbit skin and rabbit eye.
A conclusion on sensitisation cannot be drawn (vehicle used is not a recommended vehicle for LLNA; the maximum test concentration is too low).

Dermal absorption
0.48% of the applied dose corresponds to 1.62 µg/cm². The mean + 1SD (1.62 + 0.75) = 2.37 µg/cm^2 should be used to calculated MoS.

Mutagenicity / genotoxicity
The *in vitro* genotoxicity of Basic Brown 17 has been investigated in valid genotoxicity tests for the three genotoxic endpoints: gene mutation, structural and numerical chromosome aberration. Basic Brown 17 induced gene mutations in bacteria. On the other hand basic Brown 17 did not induce mutations in two *in vitro* gene mutation tests in mammalian cells, a mouse lymphoma assay (*tk* locus) and a *hprt* test. However, the appropriate level of toxicity (10-20% survival after the highest dose) was not everywhere reached in the experiments which may point to insufficient exposure of the cells. Basic Brown 17 was not considered mutagenic in an *in vitro* micronucleus test. The absence of both mutagenicity and clastogenicity in mammalian cells was confirmed in a comet assay, assumed predictive for both clastogenicity and mutagenicity, in reconstructed human skin.
On the basis of a weight of evidence approach (see Addendum to the Notes of Guidance 2014) that the positive result in the gene mutation test in bacteria is not confirmed in three different tests, measuring gene mutations in mammalian cells, Basic Brown 17 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

4. CONCLUSION

SCCS considers Basic Brown 17 safe for use in non-oxidative hair dye formulations with a concentration of maximum 2.0%, apart from its possible sensitisation potential.

Basic Brown 17 may contain up to 4.5 % (w/w) Basic Red 118, corresponding to maximum 0.09% in a hair dye formulation. Basic Red 118 according to the Cosmetic Regulation (Regulation 1223/2009) is not permitted for use in cosmetics except as an impurity in Basic Brown 17.

5. MINORITY OPINION

Not applicable.

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