Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Brown 16

COLIPA n° C9

The SCCS adopted this opinion at its 17th plenary meeting

Of 11 December 2012

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.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Elsa Nielsen, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Jan van Benthem, Jacqueline van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate D: Health Systems and Products
Unit D3 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-SCCS-Secretariat@ec.europa.eu

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Prof. J. Angerer
Dr. C. Chambers
Dr. E. Nielsen
Dr. W. Lilienblum (associated scientific advisor)
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Dr. J. van Benthem (rapporteur)
Prof. M.P. Vinardell
Dr. I.R. White

External experts

Dr. Mona-Lise Binderup National Food Institute, Denmark

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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 for Basic Brown 16 with the chemical name 8-[(4-Aminophenyl)diazenyl]- 7-hydroxy-N,N,N-trimethylnaphthalen-2-aminium chloride was submitted in August 1992 by COLIPA[1].

Submission II was submitted in July 2001 by COLIPA.

Submission III was submitted in December 2005 by COLIPA.

The Scientific Committee on Consumer Products (SCCP) issued an opinion (SCCP/1165/08) in September 2008 with the conclusions that some aspect related to the genotoxicity potential of this ingredient needed clarification.

The purpose of the present submission IV, submitted by Cosmetics Europe[2] in April 2012, is to describe the results of two *in vitro* genotoxicity studies together with a safety assessment for the use of this ingredient.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Safety (SCCS) consider Basic Brown 16 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 2.0% taken into account the scientific data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use of Basic Brown 16 in non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] COSMETICS EUROPE- former COLIPA

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.2. Primary name and/or INCI name

Basic Brown 16 (INCI name)

3.1.3. Chemical names

2-Naphthalenaminium, 8-[(4-aminopheny1)azo]-7-hydroxy-N,N,N-trimethyl-, chloride
Ammonium, [8-[(p-aminophenyl)azo]-7-hydroxy-2-naphthyl]-trimethyl-, chloride
8-[(4-Aminophenyl)diazenyl]-7-hydroxy-*N*,*N*,*N*-trimethylnaphthalen-2-aminium chloride

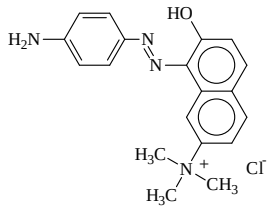
3.1.4. Trade names and abbreviations

Arianor Mahogany
Arianor Mahogany 306002
C.I. 12250
C.I. Basic Brown 16
COLIPA C 009

3.1.5. CAS / EC number

CAS: 26381-41-9
EINECS: 247-640-9

3.1.6. Structural formula



3.1.7. Empirical formula

Formula: $C_{19}H_{21}N_4O^+ Cl^-$

3.1.8. Physical form

Dark green to black powder

3.1.9. Molecular weight

Molecular weight: 356.86, calculated as HCl

3.1.10. Purity, composition and substance codes

From SCCNFP/0668/03 (adopted during the 23rd plenary meeting of 18 March 2003

Composition:	Dye (as chloride)	73.1%
	Sugar	15.1%
	volatile matter/water of crystallisation	6.1%
	inorganic salts (chloride, sulphate, etc.)	to 100%
Purity of the dye (batch: Lot 7)		>9 4 area% (HPLC)

Purity and Composition of material used in the market

Purity by NMR assay:	> 85% (w/w)	
Purity by HPLC assay:	> 97% (area)	
Chloride:	< 14% (w/w)	[NaCl: 3.4%]
Sulphated ash:	< 7% (w/w)	[corresponds to 8.6% NaCl]
7-Hydroxy-N,N,N-trimethyl-naphthalen-2-aminium chloride	< 0.5% (w/w)	
Heavy Metals (ppm):	Pb<20, Sb<10, Ni<10, As<5, Cd<5, Hg<1	
Solvent content (water):	< 6% (w/w)	
Methylchloride *:	< 2.5% (w/w)	
Methylbromide *:	< 1.2% (w/w)	

Before marketing of Basic Brown 16, sodium chloride and/or saccharose are usually added to the neat dye in order to adjust the colour strength to a certain predefined value.

* Methylbromide is classified by the EU as a mutagen category 2
Methylchloride is classified by the EU as a carcinogen category 2

Ref: 1

Batch 0095424101 (analytical report was not submitted, only results were provided)

Identification by NMR	
NMR purity:	88.6% (calculated as chloride)
HPLC content (254 nm):	≥99% (HPLC peak area)
Chloride content:	12.47% (w/w)
Water content:	4.8% (w/w)
p-Phenylenediamine:	17 ppm
4-aminoacetanilide:	2ppm
Bandrowski's base:	5 ppm
Monomethyl sulphate:	0.4%
Sodium chloride:	5.1%

Analytical description of Batches used in Toxicity studies

Batch	57861/2=SAT 050020	12/13 =SAT 040268	KS 6024
Reference	*(2)*	*(3), (4)*	*(5)*
Identity: verified by	NMR, IR and UV	NMR, IR and UV	IR and UV
Purity by NMR assay:	89.1% (w/w) *	65.7% (w/w) *	65.7%
Purity by UV/VIS			50-60% (w/w)
Purity by HPLC assay: (area, at pH 6.7) (area, at pH 2)	99.5% - -	98.9%(3) 97.9% - -	 97.5% 93.4%
Solvent content (water):	4.8% (w/w)	4.2% (w/w)	4.2% (w/w)
Methylsulfate:	not determined	0.8% (w/w)	not analysed
Methylchloride **	2.0% (w/w)	2.9% (w/w)	not analysed
Methylbromide **	1.0% (w/w)	not analysed	not analysed
NTBRI (7-Hydroxy-N,N,N-trimethyl-naphthalen-2-aminium chloride) (w/w)	0.13%	not analysed	0.9% (w/w)
Unidentified impurities:	~0.5% (~10 peaks)		1.2%

Batch		57861/2=SAT 050020	12/13 =SAT 040268	KS 6024
Chloride:	(by ion-chromatography)	11.5% (w/w)	20.3% (w/w) *	-
	(by titration)	-	-	9%
	(by HPLC)	-	-	14.6%
Sodium:		1.5 %	7% (w/w) *	not analysed
Saccharose				14.6% (w/w)*
Sulphated ash:		5.3% (w/w)	30% (w/w) *	not analysed

* Note: The Batches 12/13 and KS 6024 represent actual market materials containing additional salts (e.g. sodium chloride) and/or saccharose as "extenders" which are used to adjust the colour strength to a predefined value.

** Methylbromide is classified by the EU as mutagenic category 3
Methylchloride is classified by the EU as carcinogenic category 3

Remark:
The Purity by NMR = 65.7% (w/w) stated in the Summary submission III for the batch KS 6024 is contradictory to the data reported in the specific analytical file (ref. 5), according to which the content is 50-60% by UV/VIS analysis using the extinction coefficients calculated for the batch 57861/2.

Other batches *(statement by the authors)*
The batch 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 the specification of the contained dye is quite similar to the fully characterized batch 57861/2.

Ref: 1

Comment
The Purity by NMR = 65.7% (w/w) stated in the Summary submission III for the batch KS 6024 is contradictory to the data reported in the specific analytical file (ref. 5), according to which the content is 50-60% by UV/VIS analysis using the extinction coefficients calculated for the batch 57861/2.

The NOAEL of the 90-day and teratogenicity study needs to be corrected according to the dye content.

3.1.11. Impurities / accompanying contaminants

In addition to the NTBRI (7-Hydroxy-N,N,N-trimethyl-naphthalen-2-aminium chloride) mentioned in the previous section 3.1.4, at least 10 additional impurities were detected (but not identified) by HPLC in batch 57861/2, representing about 0.5%.

Comparable HPLC data are not provided for the other batches, but the number of additional non-identified impurities may be more taking into account that their total content is 1.2%. Also, the content of NTBRI in the batch KS 6024 was found much higher than in the batch 57861/2:

batch 57861/2	Basic Brown 16 = 89.1%	NTBRI = 0.13%	(0.13/89.1 = 0.0015)
batch KS 6024	Basic Brown 16 = 50-60%	NTBRI = 0.90%	(0.90/60.0 = 0.0150)

3.1.12. Solubility

Water:	373 g/L at 20°C	EU Method A.6
Ethanol:	10-100 g/l at room temperature	
DMSO:	50-200 g/l at room temperature	

3.1.13. Partition coefficient (Log Pow)

Log Pow: -0.74 (pH 9.95, 23°C) EU Method A.8
Log Pow: 0.88 (calc.)

3.1.14. Additional physical and chemical specifications

Appearance:	Dark green to black powder
Melting point:	169-175 °C (in SCCNFP/0668/03 it was 160-170 °C-decomposition)
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum:	(200-800 nm): λmax 218 nm, 259 nm, 478nm

3.1.15. Homogeneity and Stability

No data provided

General Comments on physico-chemical characterisation

* Impurities in the dye have not been characterised; at least 10 additional non-identified impurities were detected by HPLC.
* Unidentified impurities in batch KS 6024 at a concentration of 1.2% have not been characterised.
* Inorganic salts (5.7%) in the dye formulation have not been specified.
* the solubility has not been determined by the EU method;
* the stability data of Basic Brown 16 in the test solutions and in typical hair dye formulations were not reported.
* Methylbromide is classified by the EU (CLP) as mutagenic category 2; methylchloride is classified by the EU as carcinogenic category 2
* The cleavage of the azo-group may release para-phenylenediamine (PPD)

3.2 Function and uses

Basic Brown 16 is used as a direct dye for hair colouring products without mixing with an oxidising agent (e.g. hydrogen peroxide). The final concentration on head of Basic Brown 16 can be up to 2.0%.

3.3 Toxicological Evaluation

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

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Taken from SCCNFP/0668/03, revised

Guideline:	/
Species:	CFY rat
Group size:	2 male + 2 female rats
Material:	Basic Brown 16 in 1% aqueous methylcellulose
Batch:	/
Dose:	0, 0.1, 1.0, 2.0 and 4.0 g/kg bw in volumes of 1.0 to 40 ml/kg
Observation period:	14 days
GLP:	not in compliance
Study period:	July 1977

Groups of 2 male and 2 female rats received a single oral dose of 0.1, 1.0, 2.0 or 4.0 g/kg bw. Control animals received 1% aqueous methylcellulose in a volume of 40 ml/kg. The animals were observed daily for 14 days for mortality and clinical abnormalities. Body weights and macroscopic observations were recorded, but histological examinations were not performed.

Results
Within one week of dosing, all animals treated at 4.0 g/kg bw died, one female died after a dose of 1.0 g/kg and one after a dose of 2.0 g/kg bw; no male rats died at doses of 1 or 2 g/kg bw. There were no mortalities at 0.1 g/kg. Signs of reaction to treatment, observed shortly after dosing, included piloerection and abnormal body carriage (hunched posture). The bodyweight gain of surviving treated animals was similar to controls and no abnormalities were recorded at autopsy.

Conclusion
The LD50 was reported to be between 2 and 4 g/kg bw.

Ref.: 6

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 (2002)
Species:	New Zealand white rabbit, SPF
Group:	3 (1 male and 2 females)

Test substance:	C 009
Batch:	12/13
Purity:	98.9% (HPLC)
Dose:	0.5 g of C 009 moistened with 0.1 ml of purified water (pH 5.45)
Vehicle:	purified water
GLP:	in compliance
Study period:	11 – 24 May 2004

The test substance was applied by topical semi-occlusive application of 0.5 g to the intact left flank of each of three young adult New Zealand White rabbits. The duration of treatment was four hours. The scoring of skin reactions was performed 1, 24, 48 and 72 hours after removal of the dressing. The mean score was calculated across 3 scoring times (24, 48 and 72 hours after patch removal) for each animal for erythema/eschar grades and for oedema grades, separately.

Results
The mean erythema/eschar score and the mean oedema score was 0 for all three animals. The application of C 009 to the skin resulted in no signs of irritation. However slight brown staining of the treated skin produced by the test item was observed in all animals at the 1- and 24-hour reading. No corrosive effects were noted on the treated skin of any animal at any of the measuring intervals and no clinical signs were observed. Thus, the test item did not induce significant or irreversible damage to the skin.

Conclusion
Under the conditions of the test, the study authors considered C009 to be not irritating to rabbit skin.

Ref.: 7

3.3.2.2 Mucous membrane irritation

Guideline:	OECD 405 (2002)
Species:	New Zealand white rabbit, SPF
Group:	3 (1 male and 2 females)
Test substance:	C 009
Batch:	12/13
Purity:	98.9% (HPLC)
Dose:	0.1 g of C 009 (pH 5.45)
Vehicle:	/
GLP:	in compliance
Study period:	25 May – 16 June 2004

The test substance was applied by instillation of 0.1 g into the left eye of each of three young adult New Zealand White rabbits. The treated and untreated eyes were rinsed with lukewarm tap water 48 hours after instillation. Scoring of irritation effects was performed approximately 1, 24, 48 and 72 hours, as well as 7, 10 and 14 after test item instillation. The mean score was calculated across 3 scoring times (24, 48 and 72 hours after instillation) for each animal for corneal opacity, iris, redness and chemosis of the conjunctivae, separately.

Results
The individual mean scores for corneal opacity were 0.33, 0.67 and 0.33, respectively. The individual mean scores for the iris were 0.00 for all three animals. The individual mean scores for the conjunctivae were 1.67, 2.00 and 2.00 for reddening and 1.67, 1.33 and 1.67 for chemosis, respectively.
The instillation of C 009 into the eye resulted in mild to moderate, early-onset and transient ocular changes, such as reddening of the conjunctivae and sclerae, discharge and chemosis. These effects were reversible and were no longer evident 14 days after treatment, the end

of the observation period for all animals. Slight opacity of the cornea, affecting the whole area, was noted in all animals at the 1- and 24-hour reading due to staining produced by the test item. Slight opacity of the cornea, affecting the whole area, was again visible in one animal 72 hours after treatment. No abnormal findings were observed in the iris of any animal at any of the examinations. No corrosion was observed at any of the measuring intervals. Brown staining of the treated eyes produced by the test item was observed in all animals 1 and 24 hours after treatment. No clinical signs were observed.
Thus, the test item did not induce significant or irreversible damage to the rabbit eye.

Conclusion
Under the conditions of the test, the study authors considered C009 to be not irritating to rabbit eye.

Ref.: 8

Comment
Neat Basic Brown showed irritant potential to rabbit eyes.

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)	
Species:	Mice, CBA/CaOlaHsd	
Group:	3 test and 1 control group; 4 females per group	
Substance:	C009	
Batch:	12/13	
Purity:	98.9% (HPLC)	
Dose:	test substance:	5, 10 and 25% (w/v) in ethanol:water, 7:3 (v/v)
	positive control:	5, 10 and 25% (w/v) in acetone:olive oil, 4:1 (v/v)
Vehicle:	test substance:	ethanol:water, 7:3 (v/v)
	positive control:	acetone:olive oil, 4:1 (v/v)
Control:	α-hexylcinnamaldehyde	
GLP:	in compliance	
Study period:	5 – 19 May 2004	

Three groups of four female mice were treated daily with the test item at concentrations of 5, 10 and 25% (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. 25% (w/v) was the highest technically applicable concentration in the chosen vehicle. A control group of four mice was treated with the vehicle only. 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.

Results
All treated animals survived the scheduled study period. No clinical signs were observed. The results obtained (Stimulation Index (S.I.)) are reported in the following table. The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

Concentration % (w/v)	Test substance	Positive control
5	1.6	1.5
10	2.6 *	2.3 *
25	5.3 *	8.4 *
EC3	12.2%	11.7%

* Value used for the calculation of EC3

Conclusion
The study authors considered C 009 to be a skin sensitizer under the conditions of the test. An EC3 value of 12.2% was derived.

Ref.: 9

Comment
C 009 was shown to be a moderate skin sensitiser.

3.3.4 Dermal / percutaneous absorption

Guideline:	OECD 428 (2004)
Tissue:	dermatomed pig skin, 560 to 950 µm thickness
Group size:	2 donors (1 male, 1 female)
Diffusion cells:	8 static penetration cells (Franz-cells, 1.0 cm² application area) per experiment
Skin integrity:	transcutaneous electrical resistance (TER), TER ≥ 7 kΩ
Test substance:	C 009 (8[(E)-(4-aminophenyl)diazenyl]-7-hydroxy-N,N,N-trimethyl-naphthalen-2-aminium chloride)
Batch:	57861/2
Purity:	99.5% (area%, HPLC)
Test item:	experiment A: 2% C 009 in a direct dye formulation TM0039-1a Experiment B: 2% C 009 dissolved in water
Doses:	20 mg/cm², or 0.4 mg/cm² of test substance
Receptor fluid:	Dulbecco's phosphate buffered saline (PBS)
Solubility receptor fluid:	assumed close to that of water (100 mg/ml)
Stability:	< 2% degradation in water over a 24h period
Method of Analysis:	HPLC
GLP:	in compliance
Date:	10 October – 6 December 2005

The test substance was studied as an ingredient of a representative direct dye formulation as well as in an aqueous solution:
Experiment A: 2 % C 009 incorporated in a direct dye cream.
Experiment B: 2 % C 009 dissolved in water.

Eight integrity checked dermatomed skin preparations of two young pigs of both sexes were used in each experiment. Skins were inserted in static penetration cells (Franz-cells) with an application area of 1.0 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.
The test substance formulation/solution was applied topically to the horny layer of the skin in nominal quantities of 20 mg/cm², which corresponded to nominally 0.4 mg of the test substance per cm² for each experiment.
48 hours after the application, the *stratum corneum* was removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin was taken to determine the absorbed test substance. The penetration was calculated from the mass of the test substance in the receptor fluid, consisting of phosphate buffered saline. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

Results
The means of test results are presented in the following table:

Parameter	Experiment A		Experiment B	
	µg/cm²	%	µg/cm²	%
Skin rinsings	310	82.8	250	58.9
Adsorption	9.9	2.67	9.6	2.27
Absorption	5.1	1.41	4.5	1.06
Penetration	0.055	0.0154	0.061	0.0144
Bioavailability	**5.15 (2.19 – 9.12)**	**1.42 (0.58 – 2.91)**	**4.57 (3.76 – 5.80)**	**1.08 (0.89 – 1.37)**
Mass balance *	/	87.1	/	62.6

* These low values, slightly below the official required minimum of 85% recovery, are due to the persistency of the test substance bound to the pads and filters.

The individual test results are presented in the following tables:

Experiment A: 2% C 009 in a direct dye formulation TM0039-1a

	µg/cm²								
Skin sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Mean**
Adsorption	11.936	11.889	9.078	7.885	11.885	6.029	12.729	7.998	***9.9 ± 2.5***
Absorption	2.732	7.390	4.030	2.139	5.150	4.460	8.990	5.860	***5.1 ± 2.3***
Penetration	0.043	0.064	0.009	0.048	0.030	0.043	0.134	0.066	***0.055 ± 0.037***
Bioavailability	2.775	7.454	4.039	2.187	5.180	4.503	9.124	5.926	***5.15 ± 2.32***
Skin rinsings	375.26	294.06	312.64	324.23	309.27	312.62	245.01	307.14	***310 ± 36***

	%								
Skin sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Mean**
Adsorption	2.8172	3.2345	2.4221	2.0737	2.8916	1.6734	4.0637	2.2086	***2.67 ± 0.75***
Absorption	0.6447	2.0106	1.0753	0.5627	1.2530	1.2378	2.8701	1.6182	***1.41 ± 0.76***
Penetration	0.0102	0.0174	0.0025	0.0126	0.0073	0.0120	0.0429	0.0183	***0.015 ± 0.012***
Bioavailability	0.6549	2.0280	1.0777	0.5752	1.2604	1.2498	2.9130	1.6366	***1.42 ± 0.77***
Skin rinsings	88.570	80.003	83.417	85.273	75.246	86.764	78.218	84.818	***82.8 ± 4.6***
Balance	92.101	85.365	87.060	88.027	79.554	89.899	85.771	88.710	***87.1 ± 3.7***

Experiment B: 2% C 009 dissolved in water

	µg/cm²								
Skin sample	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**Mean**
Adsorption	11.483	13.274	13.628	10.935	10.718	5.159	6.734	5.251	***9.6 ± 3.4***
Absorption	4.622	3.718	4.960	3.960	5.710	4.866	3.978	4.259	***4.5 ± 0.7***
Penetration	0.027	0.047	0.090	0.041	0.090	0.061	0.064	0.070	***0.061 ± 0.022***
Bioavailability	4.649	3.765	5.049	4.002	5.800	4.927	4.043	4.329	***4.57 ± 0.67***
Skin rinsings	291.39	251.72	256.16	246.42	245.92	257.04	233.20	219.19	***250 ± 21***

	%								
Skin sample	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**Mean**
Adsorption	2.7080	3.1458	3.1982	2.5788	2.5398	1.2107	1.5503	1.2504	***2.27 ± 0.82***
Absorption	1.0899	0.8811	1.1639	0.9340	1.3532	1.1419	0.9158	1.0143	***1.06 ± 0.16***
Penetration	0.0063	0.0111	0.0211	0.0097	0.0214	0.0143	0.0148	0.0166	***0.014 ± 0.005***
Bioavailability	1.0962	0.8922	1.1850	0.9437	1.3746	1.1562	0.9306	1.0309	***1.08 ± 0.16***
Skin rinsings	68.716	59.652	60.115	58.112	58.277	60.322	53.685	52.199	***58.9 ± 5.0***
Balance	72.730	64.034	64.703	61.723	62.712	62.916	56.618	54.755	***62.5 ± 5.4***

Conclusion
In the direct dye formulation, the amount considered to be absorbed was 5.15 µg/cm² (range 2.19 to 9.12) or 1.42% of the applied dose (range 0.58% to 2.91%).

At 2% dissolved in water, the amount considered to be absorbed was 4.57 μg/cm² (range 3.76 to 5.80) or 1.08% of the applied dose (range 0.89% to 1.38).

Ref.: 17

Comment
As too few test chambers and few donors (8 chambers from 2 donors) were used in this study, the mean + 2 SD (5.15 + 2 x 2.32 = 9.79 μg/cm²) should be used for calculating the MOS.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (28 days) oral toxicity

No data submitted

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

Taken from SCCNFP/0668/03

Guideline:	OECD 408
Species:	Sprague Dawley CD rat
Route:	oral
Group size:	10 male and 10 female rats
Material:	commercial grade Basic Brown 16 in aqueous solution
Batch:	KS6024
Purity:	not stated (according to batch information, only 65.7% dye content according to NMR)
Dose levels:	0, 50, 150 and 450 mg/kg bw/day in a volume of 10 ml/kg
Exposure:	5 days per week for 13 weeks
GLP:	pre-GLP; quality assured
Date:	7 May – 12 September 1984

Basic Brown 16, in aqueous solution, was administered by oral gavage 5 days per week to groups of 10 male and 10 female rats at doses of 50, 150 and 450 mg/kg bw/day for 13 weeks. An additional 5 males and 5 females were treated at the same doses then maintained without treatment for observation in a 4-week recovery period. Controls received the vehicle only. The following investigations were performed: daily observations, bodyweights, food consumption, ophthalmoscopy, haematology and clinical chemistry, urinalysis, gross pathological examination, organ weight determination and histopathology.

Results
The dose of 450 mg/kg bw/day resulted in a marked decrease in body weight gain for both male and female animals. The decrease in males was significant from the first week of treatment and resulted in a mean bodyweight of 87% of controls at the termination. In females, the decrease was significant from week 6, with a mean bodyweight of 93% of controls at termination.
Additional signs of toxicity at the high dose were abnormal gait, abdominal position and neurotoxic symptoms. Macroscopic and histological evaluation revealed discoloration of the inner organs. At 150 mg/kg bw/day, there was a decrease in body weight gain in male animals (again from week 1, with a terminal mean bodyweight of 93% of control), but no other toxicological effects were reported. Coloured urine was excreted by animals treated with 150 and 450 mg/kg bw/day, throughout the treatment period.

Conclusion
The dose of 50 mg/kg bw/day was tolerated without any signs of adverse effects and is regarded to be the NOAEL.

Ref.: 15

Comment
As the dye content of the test substance is only 65.7%, the NOAEL value is re-calculated to be 33 mg/kg/bw/day.

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 Test

Guideline:	OECD 471 (1997)	
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	
Replicates:	triplicates in 2 individual experiments both in the presence and absence of S9-mix.	
Test substance:	C 009	
Solvent:	deionised water	
Batch:	57861/2	
Purity:	99.5 area% (HPLC) *Comment applicant on purity:* *The given value for the purity of the test substance used in the Ames test (Batch 57861/2) should become 99.5 are% (HPLC) as correctly given in the batch-related analytical report R 0500242 (Ref 2 of SMII in the corresponding SCCS opinion). The mentioned 98.8% are incorrectly referenced in the study report of this gene mutation study.*	
Concentrations:	Experiment I:	0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix for TA98 and TA100 0, 1, 3, 10, 33, 100, 333, 1000 and 2500 µg/plate without and with S9-mix for TA102, TA1535 and TA1537
	Experiment II:	0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix.
Treatment:	Experiment I:	direct plate incorporation without and with S9-mix
	Experiment II:	pre-incubation method with 60 minutes pre-incubation and at least 48 h incubation without and with S9-mix
GLP:	in compliance	
Study period:	17 February – 15 March 2005	

C 009 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 results of a pre-experiment for toxicity and mutation induction 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 clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in all strains used, the pre-experiment is reported as experiment I. Experiment I was performed with the direct plate incorporation method; experiment II with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results

Toxic effects evident as reduction in the number of revertants were observed at the higher concentrations without and with S9-mix in both experiments for all strains.
In experiment II, a more or less concentration dependent increase in the number of revertants was seen up to the toxic concentration in TA98 and TA1537 in the presence of metabolic activation. Except for these two positive findings a biologically relevant increase in revertant colonies was not found in any other tester strain.

Conclusion
Under the experimental conditions used C 009 was mutagenic in this gene mutation tests in bacteria with pre-incubation in strains TA98 and TA1537 in the presence of metabolic activation.

Ref.: 10

In vitro Mammalian Cell Gene Mutation Test (_tk_-locus)

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	duplicates in 2 independent experiments
Test substance:	C 009
Solvent:	deionised water
Batch:	57861/2
Purity:	99.5 area% (HPLC) *Comment applicant on purity:* *The same comment as given for this Batch in the Ames test.* *99.5 are% (HPLC) as correctly given in the batch-related analytical report R 0500242 (Ref 2 of SMII in the corresponding SCCS opinion).* *The mentioned 98.8% are incorrectly referenced in the study report of this gene mutation study.*
Concentrations:	Experiment I: 0, 27.5, 55, 110, 165 and 220 µg/ml without S9-mix 0, 55, 110, 165, 220 and 330 µg/plate with S9-mix Experiment II: 0, 55, 110, 165, 220 and 330 µg/plate with S9-mix
Treatment	4 h treatment without and with S9-mix; expression period 72 h and selection period of 10-15 days
GLP:	in compliance
Study period:	8 February – 11 April 2005

C 009 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. In the main tests, cells were treated for 4 h followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Toxicity was measured in the main experiments as percentage total growth of the treated cultures relative to the total growth of the solvent control cultures. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
The recommended toxic range of approximately 10-20 % survival compared to the concurrent negative controls was covered in all experiments.
In experiment I without metabolic activation a concentration dependent and biologically relevant increase in the mutant frequency was observed. This increase appeared to be the result of an increase in the number of small colonies. In experiment I with metabolic activation an increase in the mutant frequency was found in one of two cultures. Nor in the second culture of experiment I nor in experiment II with metabolic activation this positive finding could be confirmed.

Conclusion
Under the experimental conditions used, C 009 induced mutations in mammalian cells in the absence of metabolic activation. The results indicate clastogenic potential of C 009, because of the induction of small colonies.

Ref.: 11

In Vitro Gene Mutation Test in V79 cells (*hprt* locus)

Guideline:	/
Cells:	V79 cells
Replicates:	triplicates in 2 independent experiments
Test substance:	Arianor Mahogany (Basic Brown 16)
Solvent:	phosphate buffered saline
Batch no.:	KS 6024, Williams
Purity:	73.1 % (as chloride)
Concentrations:	0, 5, 19, 20, 50 and 100 µg/ml without S9-mix. 0, 100, 300, 1000, 3000 and 6000 µg/plate with S9-mix
Treatment	2 h treatment with S9-mix or 20 h without S9-mix; expression period 5 days and selection period of 7 days
GLP:	in compliance
Study period:	21 March – 20 July 1991

Arianor Mahogany was assayed for gene mutations at the *hprt* locus of V79 cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from Aroclor-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of an initial toxicity test measuring cell survival 5 days after treatment. In the main tests, cells were treated for 2h with S9-mix or for 20h without S9-mix followed by an expression period of 5 days to fix the DNA damage into stable *hprt* mutations. Data on toxicity were restricted to those on cloning efficiency after the selection period. Negative and positive controls were included.

Results
The data on cloning efficiency after the selection period did not indicate strong cytotoxicity; the required 10-20% survival after the highest concentration was not reached at any concentration.
In both experiments a biologically relevant and concentration dependent increase in the mutant frequency was not observed, neither in the presence nor in the absence of metabolic activation. Occasionally, an increase in mutant frequency was found; these were not reproducible and considered not biologically relevant.

Conclusion
Under the experimental conditions used, Arianor Mahogany did not induce gene mutations in this gene mutation test in mammalian cells and, consequently, Arianor Mahogany is not mutagenic in V79 cells.

Ref.: 12

Comment
As data on toxicity are restricted to those on cloning efficiency after the selection period and thus reliable data on exposure are lacking, the value of this gene mutation test in mammalian cells is limited.

In vitro Micronucleus Test

Guideline:	draft OECD 487 and accepted scientific/regulatory principles of OECD 487
Cells:	V79 cells
Replicates:	duplicate cultures
Test substance:	C 009
Solvent:	deionised water
Batch:	57861/2
Purity:	99.5 area% (HPLC) *Comment applicant on purity:* *The same comment as given for this Batch in the Ames test.*
Concentrations:	0, 450, 900, 1800 and 3600 µg/ml without S9-mix 0, 450, 900 and 1800 µg/ml with S9-mix
Treatment	4 h treatment; harvest time 24 hours after the beginning of treatment
GLP:	in compliance
Study period:	19 April – 21 June 2005

C 009 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 C 009, 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. 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. In parallel to the micronucleus test, for assessment of cytotoxicty a XTT test was carried out. Negative and positive controls were in accordance with the draft guideline.

Results
Both in the pre-test and the main test precipitation of C 009 was observed with 1800 µg/ml and above in the presence and absence of S9-mix. Clear toxic effects indicated by reduced cell numbers below 40% of control were observed after 4 h treatment with 3600 µg/ml in the absence of S9-mix and with 1800 µg/ml in the presence of S9-mix. The XTT test did not point to cytotoxicity up to the highest concentration tested (3600 µg/ml).
In the absence of S9-mix a biologically relevant but not concentration dependent increase in cells with micronuclei was observed. The number of micronucleated V79 cells slightly exceeded the historical control data range. In the presence of S9-mix a clear concentration dependent and biologically relevant increase in micronucleated cells was observed.

Conclusion
Under the experimental conditions used C 009 induced an increase in micronucleated cells and, consequently, is clastogenic and/or aneugenic in V79 cells.

Ref.: 13

Comment
Since C 009 was genotoxic after 4h treatment, a second experiment was not required according to international guidelines.

New study, submission III

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)
Cells:	L5178Y mouse lymphoma cells (*hprt* locus)
Replicates:	duplicate cultures in two independent experiments
Test substance:	C009
Batch:	57861/2

Purity:	99.5% (HPLC)
Solvent:	sterile purified water
Concentrations:	Experiment I: 0, 20, 40, 60, 80, 100, 115, 130, 145, 160 and 180 µg/ml without S9-mix 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 and 250 µg/ml with S9-mix
	Experiment II: 0, 30, 60, 80, 100, 120, 140, 160, 180, 200 and 220 µg/ml without S9-mix 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 µg/ml with S9-mix
Treatment:	3 h treatment without and with S9-mix; expression period 7 days and selection period of 12 days
GLP:	in compliance
Study period:	4 June 2009 – 29 July 2009

C009 was assayed for gene mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a cytotoxicity range-finding experiment measuring relative survival. Concentrations ranging up to 5000 µg/ml (in excess of 10 mM at the highest concentration tested) were tested. In the main tests, cells were treated for 3h without and with S9-mix followed by an expression period of 7 days to fix the DNA damage into stable *hprt* mutations. Toxicity was measured in the main experiments 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 draft guideline.

Results
In the cytotoxicity range-finder the highest concentration to provide approximately 10% relative survival or above, was found at 156.3 µg/ml, which gave 10% and 24% relative survival in the absence or presence of S9-mix, respectively. The appropriate levels of toxicity (10-20% relative survival after the highest concentration) were also reached in experiment I and II without and with S9-mix.
In both experiments, a concentration dependent and statistically significant increase in the number of mutant colonies was observed both in the presence and absence of S9-mix.

Conclusion
Under the experimental conditions used, C009 was mutagenic in this gene mutation assay with mouse lymphoma cells using the *hprt* locus as reporter gene.

Ref.: 1 (subm III)

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

Guideline:	/
Tissue:	Epiderm™ reconstructed human skin tissue
Replicates:	4 tissues/concentration in 3 independent experiments
Test substance:	Basic Brown 16
Batch:	batch 1: 8-778 (concentration range-finder experiment) batch 2: 95424101
Purity:	batch 1: >95% (concentration range-finder experiment) batch 2: > 99.0% (HPLC at 254 nm)
Solvent:	ethyl alcohol
Concentrations:	3.13, 6.25, 12.5, 25 and 50 µg/tissue
Treatment:	3h treatment
GLP:	/
Study period:	26 April 2011 – 19 July 2011

Basic Brown 16 has been investigated for induction of DNA damage in reconstructed human skin tissue using the Comet assay. The reconstructed skin tissue consists of normal, human derived epidermal keratinocytes which have been cultured to form a multilayered, metabolically and mitotically active differentiating model of the human epidermis. Ultrastructurally the reconstructed skin tissue consists of organised basal, spinous, granular and cornified layers analogous to those found in human skin, with a functioning stratum corneum which reproduces the barrier function of the skin. The initial concentrations were chosen based on solubility assessment of Basic Brown 16. The actual concentrations used in the main experiment were based on the results of a concentration range-finder experiment, with concentrations ranging up to 100 µg/tissue. In the main experiment the concentration was adjusted due to precipitation at the highest concentration in the concentration range-finder.
Before treatment, each tissue was visually inspected and observations of defects like blisters, raised edges, tissue detachment and excessive moisture was recorded. Basic Brown 16 was added in 10 µl in 100% ethanol on top of the tissue.
Tissues were treated for 3 h. Cell suspensions were obtained with 0.25% trypsin solution. Electrophoresis was performed for 30 min at 39 V, after adjustment of the amperage to 450 ± 50 mA. DNA was stained with the fluorescence dye SYBR Gold. For the evaluation of Comets the % tail DNA (= tail intensity) was used as assessment parameter. 50 cells per slide, 3 slides per tissue and 4 tissues per concentration were scored. Cytotoxicity was measured as relative cell number and relative cell viability. Appropriate negative and positive controls were included.

Results
In the concentration range-finder precipitation was observed at the 2 highest concentrations 50 and 100 µg/tissue. Consequently, it was decided that 50 µg/tissue would be the highest concentration in the main experiment. In all main experiments, no precipitation was observed at the beginning of the incubation period; however, at the end of treatment a visible dark ring was observed around the edge of the tissues at 25 and 50 µg/tissue. In all main experiments, the relative cell viability in Basic Brown 16 treated tissues was not decreased compared to the solvent controls. Basic Brown 16 is thus not cytotoxic at these concentrations.
In experiment 1, a biologically relevant increase in the % tail DNA was not found. The % tail DNA values fell within the range of the concurrent solvent control. In experiment 2, Basic Brown 16 did induce a statistically significant increase in the % tail DNA at 2 concentrations, 12.5 and 50 µg/tissue. However, the increases were of questionable biological relevance as a concentration-response was lacking. The experiment was repeated in experiment 3. As in experiment 1, a biologically relevant increase in the % tail DNA was not found in experiment 3. The % tail DNA values again fell within the range of the concurrent solvent control.

Conclusion
Under the experimental conditions used, Basic Brown 16 was not genotoxic in this *in vitro* alkaline Comet assay with reconstructed human skin tissue.

Ref.: 2 (subm III)

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI
Group size:	5 mice/sex/group
Test substance:	C 009
Batch:	57861/2
Purity:	99.5 area% (HPLC)
	Comment applicant on purity:

The same comment as given for this Batch in the Ames test.

Dose level:	0, 18.75, 37.5 and 75 mg/kg bw
Route:	i.p.
Vehicle:	deionised water
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only
GLP:	in compliance
Study period:	25 July – 27 September 2005

C 009 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based in a pre-experiment on acute toxicity at various intervals of 1, 2-4, 6, 24, 30 and 48 h after start of treatment. In the main experiment mice were exposed i.p. to 0, 18.75, 37.5 and 75 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 total erythrocytes (PCE/TE). The mice of the high dose group were examined for acute toxic symptoms at intervals of around 1, 2-4, 6 and 24 h after treatment. Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/TE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the a pre-experiment on acute toxicity with exposure up to 100 mg/kg bw C 009, reduction of spontaneous activity, ruffled fur and dark red-brow urine was found up to 6 h after administration. In the main experiment identical toxic signs were found at the highest doses.
Treatment with C 009 did not result in a decreased PCE/TCE ratios compared to the untreated controls indicating that C 009 had no cytotoxic properties in the bone marrow. However, the acute toxicity and particularly the colored urine, indicate systemic availability of C 009.
A statistically significant increase in the number of cells with micronuclei was found in the low and high dose groups after 24 h exposure. However, these values are well within the historical control range and thus do not bear a biological relevance.

Conclusion
Under the experimental conditions used C 009 did not induce a biologically relevant increase in the number of micronucleated PCEs in bone marrow cells of treated mice and, consequently, C 009 is not regarded clastogenic and/or aneugenic in bone marrow cells of mice.

Ref.: 14

3.3.7 Carcinogenicity

No data submitted

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data submitted

3.3.8.2 Teratogenicity

Guideline:	OECD 414 (2001)
Species/strain:	Rat, Han Wistar, HsdBrl: female
Group size:	25 per dose
Test substance:	C009
Batch:	12/13

Purity:	98.9% (according to batch information, only 65.7% dye content according to NMR)
Dose:	0, 45, 90, 180 ml/kg bw/day
Solvent:	Distilled water
Route:	Oral gavage
Exposure:	Gestation Day (GD) 5 - 19
GLP:	in compliance
Date:	15 July – 9 August 2005

Gestation Day (GD) 0 or mating day was considered as either the presence of sperm in the vaginal smear or by the presence of a copulation plug. Doses were based on the results of the previously performed studies (not identified). The animals were observed daily; clinical signs and deaths were recorded. Body weight gain was recorded on GD 0, 5, 9, 12, 15 and 20. The dams were killed on GD 20 for *post mortem*. The number of alive and dead foetuses, their distribution and site in the uterus, early and late resorption, implantation and number of corpora lutea was determined. The weight of the foetuses, gravid uteri, uteri without foetuses, placentae and the sex of foetuses were recorded. Approximately one-half of the foetuses were selected at random and examined for visceral alterations. The remaining foetuses were examined for skeletal malformations, variations and retardation of the normal organogenesis after appropriate staining.

Results
No mortality occurred during the study. Three females, two in the control and one in the low dose group were found not pregnant. One female in the low dose group showed unilateral total resorption. In addition, two females, one in the control group and one in the mid-dose group, showed unilateral implantation. On GD 20 there were 23 females with live foetuses in the control and low dose groups and 25 in the mid and high dose groups.
Brown staining on the skin and tail was observed in the high dose group and the cage tray was stained a light pink. These were considered to be a direct result of the colour of the test substance and of no biological relevance.
Daily pre- and post-dose observations showed no treatment-related reactions. No significant differences were noted in terminal body weight, uterus weight and absolute weight gain between control and treated groups. However, on GD 9, body weight gain in the high dose group showed a statistically significant reduction compared with controls.
No treatment-related macroscopic changes were seen in dosed females.
Small foetuses (body weights <2.7 g), 37 in total, were found in all groups (control group 4 in 4 litters; low dose group 6 in 4 litters; mid-dose group 11 in 5 litters; high-dose group 16 in 9 litters). Statistically significant lower mean foetal weight was noted in the high dose group compared with the controls but this was considered to be due to the higher incidence of small foetuses in the high dose group. There was also an increased frequency of total implantation loss in the high dose group. However, this was not considered of toxicological relevance since these were mainly pre-implantation losses that could have been influenced by the factors prior to the treatment. Intra-uterine deaths were found in 16 litters of the high dose group (29 resorptions) compared to 9 litters (13 resorptions) of the control group. However, an influence of maternal toxicity in animals observed at GD 9 cannot be excluded.
Anencephaly was noted in one mid-dose foetus. In addition, another foetus from the same litter showed enlarged brain ventricles. These were considered to be incidental or spontaneous in origin.
The reduced ossification of some bones were found in a few foetuses of in all litters of treated groups, but were minimal and not dose-related. However, three foetuses from two high dose litters showed no ossification of pubis, considered to be malformations. The study authors suggested these were related to the lower foetal weight (from 30 to 47%) compared with control mean foetal weight.

Conclusion
At the high dosage of 180 mg/kg bw/day, C009 caused slight maternal toxicity as indicated by the reduction in body weight gain on GD 9 when compared with controls. As a

consequence of the maternal toxicity, a mean reduced foetal weight was observed and a delay in the ossification of some bones was noted at the skeletal examination.
Neither embryotoxic nor teratogenic effects of C009 has been found in rats under the study conditions.
The NOAEL both for maternal and embryo foetal toxicity was considered to be 90 mg/kg bw/day.

Ref.: 16

Comment
As the dye content of the test substance is only 65.7%, the NOAEL value is re-calculated to 59 mg/kg bw/day.

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

Not applicable

3.3.14 Discussion

Physico-chemical properties
Basic Brown 16 is used as a direct dye for hair colouring products without mixing with an oxidising agent (e.g. hydrogen peroxide). The final concentration on head of Basic Brown 16 can be up to 2.0%.
At least 10 additional non-identified impurities were detected by HPLC, which were not characterised. Methylbromide is classified by the EU (CLP) as mutagenic category 2; methylchloride is classified by the EU as carcinogenic category 2.
The stability data of Basic Brown 16 in the test solutions and in typical hair dye formulations were not reported. The cleavage of the azo-group may release para-phenylenediamine (PPD).

General toxicity
The acute median lethal oral dose (LD_{50}) of Basic Brown 16 was reported to be between 2 and 4 g/kg bw.
In a 90-day rat study, the NOAEL was considered to be 50 mg/kg bw/day, based on decreased body weight gain and some neurotoxic symptoms in both sexes. This value was re-calculated to 33 mg/kg bw/day based on the dye content of the test substance.
The NOAEL both for maternal and embryo-foetal toxicity was considered to be 90 mg/kg bw/day. This value was re-calculated to 59 mg/kg bw/day based on the dye content of the test substance.

Irritation / sensitisation
Basic Brown 16 was considered not to be irritating to rabbit skin. It showed irritant potential to the rabbit eye.
Basic Brown 16 was a moderate skin sensitizer under the conditions of the test. An EC3 value of 12.2% was derived.

Dermal absorption
The amount considered to be absorbed as direct dye formulation was 5.15 μg/cm² (range 2.19 to 9.12) or 1.42% of the applied dose (range 0.58% to 2.91%). At 2% dissolved in water, the amount considered to be absorbed was 4.57 μg/cm² (range 3.76 to 5.80) or 1.08% of the applied dose (range 0.89% to 1.38). As too few test chambers (8 chambers from 2 donors) the mean + 2 SD (5.15 + 2 x 2.32 = 9.79 μg/cm²) may be used for calculating the MOS.

Mutagenicity / genotoxicity
Overall, the genotoxicity of Basic Brown 16 was investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.
Basic Brown 16 did induce gene mutations in bacteria. In mouse lymphoma cells, the mutant frequency at both the *tk* as well as at the *hprt* locus was increased. In a *hprt* gene mutation test in V79 cells with limited value, an increase in the mutant frequency was not observed. The increase in the mutant frequency at the *tk* locus of the mouse lymphoma cells was due to an increase in small colonies, which in turn is indicative for a clastogenic effect of Basic Brown 16. The positive *in vitro* micronucleus test confirmed the clastogenic potency of Basic Brown 16. A Comet assay, indicative for both clastogenicity and mutagenicity, performed with reconstructed human skin was negative.
Clastogenicity found *in vitro* could not be confirmed *in vivo*. Basic Brown 16 did not induce micronuclei in an *in vivo* micronucleus test in erythrocytes of mice. The positive finding in the gene mutation tests in bacteria and mammalian cells were not confirmed nor overruled with an *in vivo* test measuring the same genotoxic endpoint. Instead of an *in vivo* gene mutation test tests a Comet assay, indicative for both clastogenicity and mutagenicity, in reconstructed human skin was performed. This *in vitro* model strongly resembles human skin and may be considered as a justified surrogate for an *in vivo* gene mutation test. The absence of DNA damage in this Comet in reconstructed human skin may indicate that the chance that Basic Brown 16 has a genotoxic potential is low. At present, this test model is not sufficiently validated. A final decision on the mutagenic potential cannot be made at the moment.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCS is of the opinion that a conclusion on the mutagenic potential of Basic Brown 16 cannot be drawn at the moment.

Characterisation of impurities in Basic Brown 16 and data on its stability in test solutions are required.

Basic Brown 16 is a moderate skin sensitiser.

5. MINORITY OPINION

Not applicable

6. REFERENCES

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